Dietary, Genetic and Metabolic Determinants of Serum Adiponectin

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Dietary, Genetic and Metabolic Determinants of Serum Adiponectin

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For Mum and Dad,

my husband Abdullah

and my kids

Fahad & Latifa

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AlSaleh A, Sanders TA, O'Dell SD (2012) Effect of interaction between *PPARG, PPARA* and *ADIPOQ* gene variants and dietary fatty acids on plasma lipid profile and adiponectin concentration in a large intervention study. *Proc Nutr Soc* **71**, 141-53.

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Abstract

The role of adiponectin, a cytokine produced in adipose tissue and its relationship to risk factors for the metabolic syndrome, and dietary influences on its production are reviewed. The thesis examines the hypothesis that the amount and type of fatty acids in the diet affect serum adiponectin concentrations. The effects of *ADIPOQ* gene locus on serum adiponectin concentrations were examined in participants of the RISCK study at baseline and following dietary intervention. Major effects of age, BMI, gender and ethnicity on serum adiponectin were found. At baseline the *ADIPOQ* +276 G/T T-allele was associated with higher serum adiponectin. The *ADIPOQ* -10066 G/A A-allele was associated with lower serum adiponectin. No influences were observed for the other SNPs -7734 C/A or -11391. Participants with the -10066 GG genotype showed a 3.8% increase and -10066 A-allele carriers showed a 2.6% decrease in adiponectin (n=360; P=0.006) after a high mononounsaturated fat diet. In-10066 GG homozygotes, adiponectin increased progressively with age after a high monounsaturated fatty acid diet but decreased after a low fat diet. A randomised single-blind parallel study in healthy males (n=48) was conducted to investigate the effects on serum adiponectin concentration of two formulations of DHA and EPA (3 g/d each) *versus* placebo (refined olive oil) for 6 weeks. Compared to the placebo, neither EPA nor DHA changed serum adiponectin concentration. It was concluded that a high monounsaturated fatty acid diet has a moderate effect on adiponectin concentration and that dietary supplements of long-chain n-3 polyunsaturated fatty acids in healthy subjects have no effect on adiponectin concentration.

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Further investigations were conducted to examine the interaction of dietary fatty acid intake with peroxisome proliferator-activated receptor (PPAR) genes. A significant interaction was found between the habitual dietary polyunsaturated/saturated fatty acid ratio and *PPARG* Pro12Ala genotype on total and LDL cholesterol and plasma triacylglycerol concentrations. After a high monounsaturated fat diet, carriages of both *PPARA* Val162 and *PPARG* Ala12 alleles were associated with a greater reduction in plasma LDL-C and its proportion as small dense LDL, than after low fat diet. A significant interactions between n-3 LCP treatment and genotypes of *PPARA* Leu162Val SNP were found among 310 participants in the MARINA trial. These contributed to a reduction in plasma triacylglycerol concentration with n-3 treatment in subjects homozygous for the more transcriptionally active Leu162 allele. The findings reported in the thesis are discussed in the context of other research in the area.

Author's contribution

The author has extracted DNA for the RISCK and MARINA studies and preformed genotyping for all SNPs. *PPARG* Pro12Ala genotyping was carried out by KBioscience.

The author and Miss Sarah Cottin (PhD student) co investigated the EPA and DHA trial, which involved recruiting and screening subjects, vascular function measurements, handling and analysis of blood samples for plasma adiponectin, glucose, insulin and plasma lipids.

The author statistically analysed and interpreted the data from all the studies and composed the present thesis. All statistical analysis was conducted in discussion with Prof. Tom Sanders, Dr. Sandra O'Dell and Dr. Peter Milligan. I am very grateful to all of them for their helpful advice and discussion throughout this work.

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Abbreviations

4-AA	4-amino-antipyrine
AA	Arachidonic acid
ACAT	Acyl:cholesterol acyl transferase
ACC	Acetyl coenzyme A carboxylase
ACOD	Acyl coenzyme A oxidase
ACS	Acyl coenzyme A synthetase
ADD1/SREBP1	Adipocyte differentiation and determination factor 1/SREBP-1
AdipoR1	Adiponectin receptor 1
AdipoR2	Adiponectin receptor 2
ALA	α-linolenic acid
AMPK	AMP-activated protein kinase
ANCOVA	Analysis of covariance
AP2	Adipocyte-specific fatty acid–binding protein gene
APS	Adenosine 5'phosphosulfate
ATP	Adenosine triphosphate
BP	Blood pressure
bp	Base pair
C/EBPα	CCAAT/enhancer binding protein-α
CAD	Coronary artery disease
CAP	c-Cbl-associated protein
CCD	Coupled device
CEU	Utah subjects of European descent
CHO	Carbohydrate
CI	Confidence interval
COX1	Cyclo-oxygenases type 1
COX2	Cyclo-oxygenases type 2
CPT-1	Carnitine palmitoyl transferase-1
CV	Coefficients of variance
CVD	Cardiovascular disease
DHA	Docosahexaenoic acid
DHLA	Dihomo-gammalinolenic acid
DINO	Diet in Nutrients out
DNA	Deoxyribonucleic acid
DNTP	Deoxynucleotide triphosphates
DPA	Docosapentaenoic acid
EDT	EPA and DHA trial
EPA	Eicosapentaenoic acid
FAS	Fatty-acid synthase
FATP	Fatty acid transport proteins
FMD	Flow mediated diameter
Fox01a	Forkhead box protein 01A
G6Pase	Gucose-6-phosphatase
GLUT4	Glucose transporter type 4
GLUT1	Glucose transporter type 1

GM	Geometric mean
GSK-3	Glycogen synthase kinase 3
HbA1c	Haemoglobin A1C
HDL	High density lipoprotein
HM	High-MUFA
HMW	High molecular weight adiponectin
HOMA-IR	Homeostatic model assessment of insulin resistance
HS	High saturated fat
IDF	International Diabetes Federation
IL-6	Interleukin-6
INSIG1	Insulin-induced gene 1
IR	Insulin receptors
IRS-1	Insulin receptor substrate-1
IVGTT	Intravenous glucose tolerance test
JNK	c-Jun NH2-terminal kinase
LCP	Long-chain n-3 PUFA
LD	Linkage disequilibrium
LDL	Low density lipoprotein
LDL-PPD	LDL peak particle diameter
LF	Low-fat
LMW	Low molecular weight adiponectin
LPL	Lipoprotein lipase
LRH-1	Liver receptor homolog-1
MAF	Minor allele frequency
MAPK	Mitogen-activated protein kinase
MARINA	Modulation of Atherosclerosis Risk by Increasing dose of N-3 fatty Acids
MEHA	3-Methyl-N-Ethyl-N-(ß-Hydroxyethyl)-Aniline
MgCl2	Magnesium chloride
MUFA	Monounsaturated fatty acid
NCBI	National Centre for Biotechnology Information
NCEP ATP III	National Cholesterol Education Program's Adult Treatment Panel
NEFA	Non-esterified fatty acid
NFKB	Nuclear factor-KB
P:S	Polyunsaturated: saturated fat
PAP	Phosphatidic acid phosphohydrolase
PCR	Polymerase chain reaction
PDK	Phosphoinositide-dependent Kinase
PEPCK	Gluconeogenesis including phosphoenolpyruvate carboxykinase
PI3K	Phosphatidylinositol 3-kinase
PKB	Protein kinase B
PKC	Protein kinase C
PLA2	Phospholipase A2
PPARα	Peroxisome proliferator-activated receptor alpha
PPARγ	Peroxisome proliferator-activated receptor gamma
PPi	Pyrophosphate

PPRE	Peroxisome proliferator response element
PTP	Protein tyrosine phosphatase
PUFA	Polyunsaturated fat acid
RISCK	Reading, Imperial, Surrey, Cambridge, King's
RXR	Retinoid X receptor
S, SE	South and South East
SD	Standard deviation
SFA	Saturated fatty acid
Si	Insulin sensitivity
SNP	Single nucleotide polymorphisms
SREBP	Sterol regulatory element-binding proteins
T2DM	Type 2 diabetes mellitus
TAG	Triacylglycerol
TBE	Tris/Borate/EDTA
TC	Total cholesterol
TNF-α	Tumor necrosis factor-α
TRL	TAG-rich lipoproteins
TZD	Thiazolidinedione
UCP-2	Uncoupling protein-2
UV	Ultraviolet
VLDL	Very low density lipoprotein
WAT	White adipose tissue
WHO	World Health Organization
YRI	Yoruba in Ibadan, Nigeria
M	Male
F	Female

Chapter 1

Introduction

Adiponectin is a 244 amino acid plasma protein secreted exclusively by adipocytes. Adiponectin gene expression is modulated by nuclear peroxysome proliferator receptor gamma (PPARγ). Lipids are the physiological ligands for the PPAR family (Xu et al, 1999). Adiponectin is an insulin sensitising compound with anti-atherogenic, anti-diabetic, anti-inflammatory functions (Oh et al, 2007). In healthy individuals, adiponectin circulates at a high concentration in serum (7.9-11.7 µg/ml) (Liu et al, 2010). The aim of this thesis was to examine the influence of dietary lipids on adiponectin concentrations and variations in the *ADIPOQ* gene on serum adiponectin concentrations. Adiponectin concentrations are negatively associated with human obesity and are associated with the development of insulin resistance and the metabolic syndrome (Isobe et al 2005; Brochu-Gaudreau et al, 2010). Consequently, the following section will describe the metabolic syndrome and its definitions.

1.1 Metabolic syndrome

The term 'metabolic syndrome' was first coined by Reaven in his Banting lecture in 1988 to describe a cluster of features characterised by obesity (particularly central obesity, raised fasting glucose/hyperinsulinaemia, elevated plasma triacylglycerol (TAG) and low high density lipoprotein cholesterol (HDL-C) concentrations, hypertension, microalbuminuria and gout associated with an increased risk of cardiovascular disease (CVD). Most patients with Type 2 diabetes (T2D) have the metabolic syndrome and it is often referred to as a prediabetic state. Other clinical features associated with metabolic syndrome are impaired fibrinolysis, increased procoagulant activity and impaired endothelial function. The current definition of metabolic syndrome by the International Diabetes Federation (IDF) has evolved as a means of diagnosing people likely to have metabolic syndrome rather than the clinical importance of the component features. The earlier definitions by the World Health Organization (WHO) (1999) included measures of urinary microalbumin excretion and insulin concentrations. The current definition has been simplified and uses lower cut-offs for blood pressure (BP) and blood glucose (**Table 1.1**) and includes a measure of central obesity (waist circumference) if body mass index (BMI) is less than 30 kg/m^2 or BMI>30 kg/m^2 and two of the following features: impaired fasting glucose (>5.6 mmol/L), hypertension or dyslipidaemia (low HDL-C or raised fasting TAG). Obesity, physical inactivity and aging are the major factors contributing to the development of the metabolic syndrome. However, the risk of developing of metabolic syndrome may

be inherited or influenced by early life events particularly the pattern of growth *in utero* and in early postnatal life.

Table 1.1 Definitions of metabolic syndrome; WHO, National Cholesterol Education Program's Adult Treatment Panel (NCEP ATP III) and IDF. From (Alberti et al, 2006)

WHO, 1999	**NCEP ATP III, 2001**	**IDF, 2005**
Diabetes or IGT or IR, plus two or more of the following:	**Three or more of the following:**	**Central obesity plus any two of the following:**
Obesity: BMI >30 or WHR >0.9(M)>0.85(F)	BMI> 30 or waist circumference>102 cm (M), >88 cm (F)	Hypertriglyceridemia: TAG ≥1.7 mmol/L or medication.
Dyslipidemia: TAG ≥1.7 mmol/L or HDL-C <0.9 mmol/L (**M**), <1.0 mmol/L (**F**)	Hypertriglyceridemia: TAG ≥1.7 mmol/L	Low HDL-C: <1.03 (M) and <1.29 mmol/L (F) or medication
Hypertension: ≥140/90 mmHg or medication	Low HDL-C: <1.03 mmol/L (M), <1.29 mmol/L (F**)**	High blood pressure: ≥130/85 mmHg or medication
Microalbuminuria: Albumin excretion ≥20μg/min or albumin/creatine ratio ≥30 mg/g	High BP: ≥130/85 mmHg.	High fasting glucose: FPG ≥ 5.6 mmol/L or previous diagnosed diabetes.
	High fasting glucose: FPG ≥6.1 mmol/L.	

Table 1.2 Ethnic-specific values for waist circumference. From (IDF, 2006*)*

Country	**Ethnic group waist circumference (cm)**	
	Men	**Women**
Europeans	≥ 94	≥ 80
South Asians	≥ 90	≥ 80
Chinese	≥ 90	≥ 80
Japanese	≥ 80	≥ 90
Ethnic South and Central Americans	Use South Asian recommendations until more specific data are available	
Sub-Saharan Africans	Use European data until more specific data are available	
Eastern Mediterranean and Middle East (Arab) Populations	Use European data until more specific data are available	

1.1.1 Clinical significance and prevalence of metabolic syndrome

The major health risk of metabolic syndrome is the development of T2D and its complications, notably CVD (Zimmet et al, 2001). It is also linked to an increased risk of some cancers (WCRF, 2007).

The prevalence of metabolic syndrome in the European population is estimated to range between one quarter to one third of the adult male population and between a fifth and a quarter of women (Qiao, 2006). Using the NCEP-III definition, the prevalence was estimated to be 6.7% among subjects aged 20 through 29 years and 43.5% for participants aged 60 through 69 years (Ford & Li, 2008). The prevalence of metabolic syndrome has increased markedly with the increase in the prevalence of obesity, as has T2D.

It has been predicted that there will be a global epidemic of T2D particularly affecting Asia with increasing levels of affluence (Zimmett et al, 2001). All the indices appear to indicate that this is occurring (IDF and CDC). [http://www.cdc.gov/diabetes/pubs/factsheet11.htm accessed 26/09/2011].

1.1.2 The role of diet in the causation of metabolic syndrome

Insulin resistance is the central feature of the metabolic syndrome, although not necessary for its definition. It appears to arise when excess amounts of fat is stored in the wrong place i.e. in muscle or the liver. Obesity, particularly central obesity, is strongly related to the risk of developing metabolic syndrome, but not all obese people exhibit signs of metabolic syndrome. Regular physical activity appears to be protective. Among environmental factors, there is convincing evidence that maintaining a healthy weight by balancing energy intake with energy expenditure is central to the prevention and treatment of metabolic syndrome (Costacou & Mayer-Davis, 2003). There is considerably less certainty regarding the sources of macronutrients, dietary fibre, type of fat, added sugars and glycaemic index.

Prospective cohort studies show no consistent association between the source of macronutrients and weight gain or weight loss. Neither do intervention trials (Pirozzo et al, 2003). The Women's Health Study noted minimal differences in body weight and the risk of heart disease among postmenopausal women given intensive dietary advice to

decrease fat intake, as compared with those given no advice over 7.5 years of follow-up (Howard et al, 2006). However among participants who lost weight and did not regain it, there were improvements in features associated with metabolic syndrome and a reduced incidence of T2D. Currently, there is a debate as to whether high carbohydrate diets may exacerbate metabolic syndrome by increasing very low density lipoprotein (VLDL), TAG synthesis and lowering HDL-C. This is one of the reasons for the increased popularity of low carbohydrate diets. Some, but not all, prospective cohort studies show carbohydrate intake to be associated with an increased risk of metabolic syndrome (Mozaffarian et al, 2011).

Insulin resistance is a condition where normal amounts of insulin are inadequate to produce a normal insulin response (Sheng & Yang, 2008). The development of T2D initiated by insulin resistance is a multistep process with strong environmental and genetic influences (Schinner et al, 2005). The type of fat in experimental animals appears to influence insulin resistance, with saturated fatty acids (SFA) increasing insulin resistance and long chain n-3 polyunsaturated fatty acids (PUFA) decreasing it. The role of monounsaturated fat (MUFA) is less clear. It is believed that fatty acids may exert their effects by acting as ligands for nuclear hormone receptors or influencing the metabolism of nuclear transcription factors.

Several large randomised controlled trials have evaluated the effects of replacing SFA with MUFA on insulin resistance. The Kanwu study (Vessby et al, 2001) was a multicentre study comparing a SFA rich diet with a MUFA rich diet on insulin sensitivity (Si) using an intravenous glucose tolerance test (IVGTT). It reported a modest impairment of insulin sensitivity with the SFA rich diet and no change on the MUFA diet; the difference between treatments was of borderline significance (P=0.053). The LIPGENE study also used an IVGTT, reported no effect of reducing SFA on Si in weight-stable obese subjects with metabolic syndrome (Tierney et al, 2011). Replacing a high SFA diet (19% of energy) with a high MUFA (20% of energy) or a Mediterranean diet (21% of energy) for 8 weeks did not affect insulin sensitivity (Bos et al, 2010) using the euglycemic clamp method. The RISCK study, which was a multicentre six month dietary intervention study comparing isoenergetic replacement of SFA with MUFA in participants judged as being at risk of metabolic syndrome (mainly on the basis of waist circumference and fasting insulin) did not influence insulin sensitivity measured by IVGTT (Jebb et al, 2010). Consequently, the evidence from

dietary intervention studies does not support the superiority of MUFA *versus.* SFA on insulin resistance in healthy and non-diabetic participants.

The evidence with regard to PUFAs and insulin resistance is less clear, particularly with regard to dietary sources of EPA and DHA. Some prospective cohort studies (Kaushik et al, 2009; Djousse et al, 2011) have found that fish consumption and the intake of long-chain n-3 PUFA (LCP) is associated with an increased risk of T2D. However, the LIPGENE study suggests an improvement in metabolic syndrome with supplementation with LCP (Paniagua et al, 2011). This is in contrast to the Kanwu (Vessby et al, 2001) and OPTILIP studies (Griffin et al, 2006) where no effect on insulin sensitivity was seen.

Adiponectin appears to play a key role in the regulation of lipid and carbohydrate metabolism and may also have a role in regulating blood flow. Individuals with low plasma adiponectin concentrations are at increased risk of developing metabolic syndrome and T2D (Oh et al, 2007). The glitazone class of drugs, which are agonists for the PPARγ nuclear hormone receptor, promote the synthesis of adiponectin and are able to suppress the release of non-esterified fatty acids (NEFA) from adipose tissue, thus decreasing hepatic TAG synthesis as well as increasing sensitivity to insulin (Belfort et al, 2006). Some derivatives of PUFAs, such as prostaglandin J2, are ligands for PPARγ and it is believed that certain fatty acids are the normal biological ligands for PPAR receptors (Itoh et al, 2008). The influence of different PUFAs on adiponectin is of much interest and the following section describes PUFAs metabolism.

1.2 Polyunsaturated fatty acid metabolism

PUFAs are carboxylic fatty acids with two or more double bonds (**Figure 1.1**). The major dietary source of PUFA is vegetable oil but significant amounts can be provided by marine lipids (**Figure 1.2**). Virtually all of the PUFA in human diets consist of linoleic acid (18:2n-6; LA) and alpha-linolenic acid (18:3n-3; ALA) and their longer chain derivatives. Plants, but not animals have the ability to insert methylene interrupted double at positions 3 and 6 from the terminal methyl group. Although animals are unable to synthesise linoleic and linolenic acid, they can convert them into longer chain polyunsaturated fatty acids, typically of 20-22 carbon chain length. These $C_{20\text{-}22}$ LCPs appear to be physiologically important (FAO/WHO, 2010; EFSA 2010).

Linoleic acid is abundant in main seed oils whereas ALA is less widely distributed. Linoleic acid is converted via dihomo-gammalinolenic acid (20:3n-6; DHLA) mainly to arachidonic acid (20:4n-6; AA) through alternating desaturation and chain elongation reactions. AA and DHLA have important roles as a membrane component as well as being a precursor for the major family of eicosanoids (prostaglandins, leukotrienes, thromboxanes, prostacyclins) which have regulatory functions both within and between cells. AA also gives rise to anandamides which are ligands for the endocanniboid receptors, which may interact with the PPAR receptors (Hansen & Artmann, 2008). Linoleic acid, however, does have a specific function in maintaining the water permeability barrier of the skin as part of acylglycosylceramines and is the preferred substrate for making cholesteryl esters used to transport cholesterol in blood (Sanders, 1988). The minimum requirement for linoleic acid is about 1% of the dietary energy intake but optimal amounts are probably closer to 4-5%. Human diets typically provide contain 10-15 g of linoleic acid daily about 4-5% of the dietary energy (FAO/WHO, 2010; EFSA 2010).

The essential role of the n-3 series is less well established but hinges on the conversion of ALA to docosahexaenoic acid (22:6n-3; DHA) which is a major component of retinal and synaptic membranes. ALA is found in high concentrations of chloroplasts in plants. Soybean oil and rapeseed oil are the major dietary sources of ALA but small amounts are also provided by milk fat and some nuts. ALA can be converted into eicosapentaenoic acid (20:5 n-3; EPA) and DHA in man. The liver is believed to the main site of conversion (Burdge & Calder, 2005) but there is evidence that conversion occurs within the brain and other tissues i.e. testis. Fish oils are rich in dietary EPA and DHA and the consumption of these preformed LCPs has different effects on lipid metabolism from ALA (Sanders & Emery, 2003). Requirements for n-3 fatty acids are in the range of 0.2%-0.5% of the energy intake provided as ALA is about 0.5 -1 g/d. Some recent dietary recommendations have suggested a population intake of 0.25g n-3 LCP (FAO/WHO 2010; EFSA 2010).

AA, EPA and DHA are transferred preferentially into the sn-2 position of phospholipids (Flachs et al, 2009; Kopecky et al, 2009). Different phospholipid species have specificity for different PUFA. For example, AA is the dominant PUFA in phosphatidyl inositol and DHA is the dominant fatty acid in retinal ethanolamine phosphoglycerides.

Phospholipids, besides being important membrane components, act as a substrate for chemical messengers, such as eicosanoids C_{20} LCP cleaved by phospholipase A2 (PLA2) (Flachs et al, 2009) available for conversion to eicosanoids. AA is the normal substrate for cyclo-oxygenases type 1 and 2 (COX-1 and COX-2). EPA does give rise to a series of metabolites that are either less active or inactive compared with those derived from AA. DHA does not form prostaglandins but like EPA does competitively inhibit the formation of prostaglandins from AA. Eicosanoids derived from n-6 PUFA are essential for normal housekeeping functions and are usually synthesised by COX-1 but excessive eicosanoid production can be induced in response to inflammation and injury via activation of COX-2. In animals, the partial replacement of AA with EPA and DHA in membrane lipids decreases the production of eicosanoids and this can modulate processes involved in thrombosis and inflammation. Recently discovered metabolites called resolvins and neuroprotectins derived from EPA and DHA may possess an anti-inflammatory effect and provide protection against tissue damage (Flachs et al, 2009; Kopecky et al, 2009; Sanders & Emery, 2003).

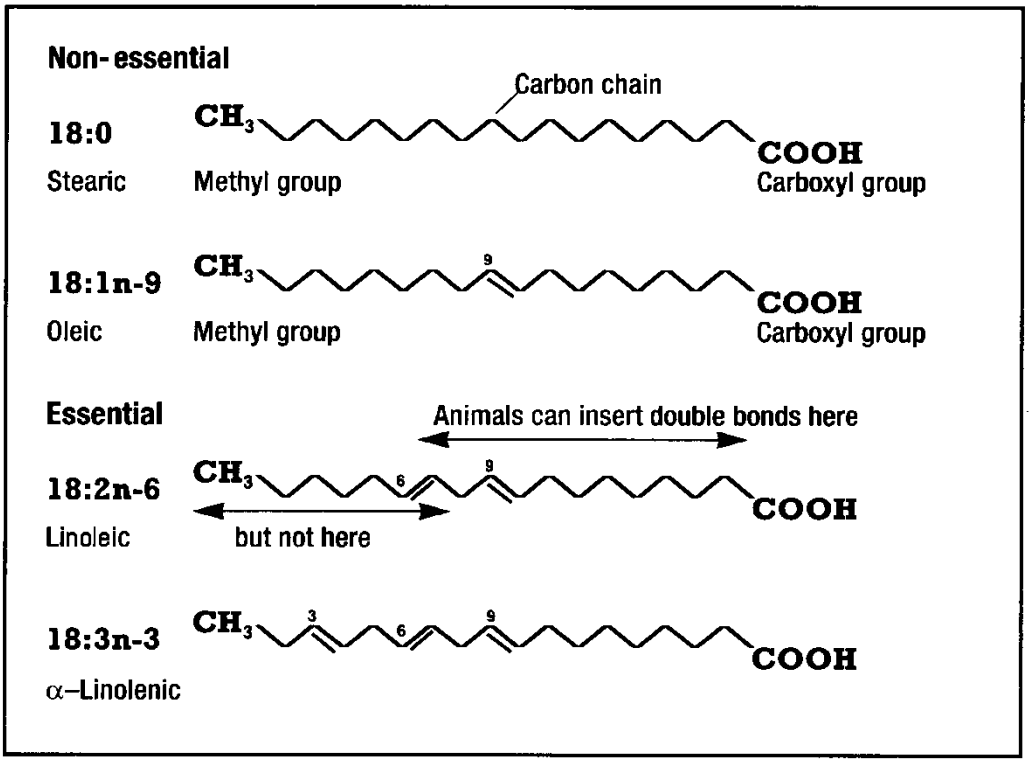

Figure 1.1 Non-essential and essential fatty acid structure. From (Sanders & Emery, 2003)

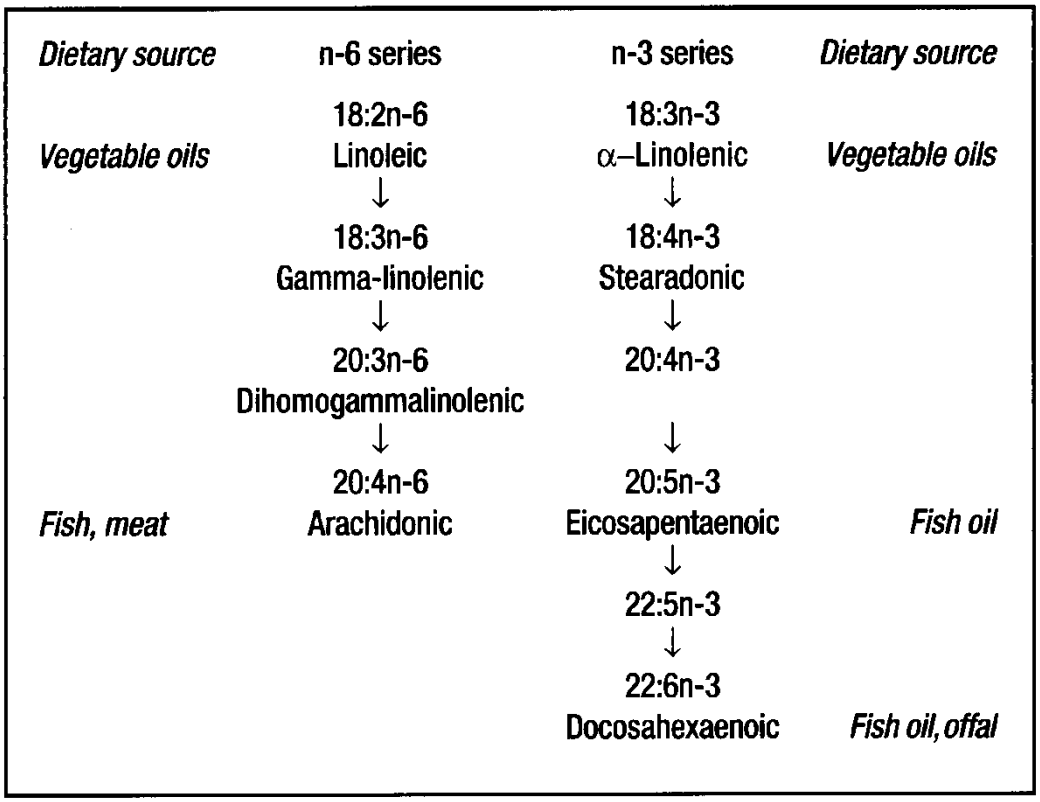

Figure1.2 Dietary polyunsaturated fatty acids. From (Sanders & Emery, 2003)

1.2.1 EPA and DHA: effect on lipid metabolism

EPA and DHA, but not ALA, lower fasting and postprandial TAG concentrations. This effect is more marked among individuals with raised plasma TAG associated with elevation of VLDL particles by the liver (Harris et al, 2008). One meta-analysis reported that the intake of 4g/d of marine omega-3 fatty acids caused a 25-30% reduction in TAG, an increase of 5 - 10 % of low density lipoprotein (LDL-C) and a 1 - 3% increase in HDL-C (Harris, 1997). Both EPA and DHA contribute to the TAG lowering effect. Variable effects have been found on LDL-C with a tendency for LDL to increase in patients with initially elevated TAG (Sanders, 2009). The mechanism which involves EPA and DHA exhibiting their effects on TAG concentrations is mainly explained by inhibiting hepatic VLDL-TAG secretion rates and decreasing TAG synthesis (Harris et al, 2008). Reduced VLDL-TAG secretion may be due to decreased expression of sterol regulatory element-binding protein-1(SREBP-1), which is a transcriptional factor involved in hepatic lipogenesis where the synthesis of acetyl-CoA carboxylase-1 (ACC1) and fatty-acid synthase (FAS) is stimulated. PUFAs found to suppress SREBP-1 expression are mediated through competition with oxysterols to bind to LXR, thereby inhibiting the binding of the LXR/retinoid X receptor (RXR) hetrodimer (which regulates the expression of SREBP-1) to the LXREs in the SREBP-1c promoter (Yoshikawa et al, 2002). Competing with LXR will also inhibit the expression of SREBP-2, which regulates the genes involved in cholesterol synthesis (Davidson, 2006).

Physiological ligands for peroxisome proliferator-activated receptor alpha (PPARα) include branched chain and C_{20-22} fatty acids. Both EPA and DHA are ligands for PPARα and this may explain why these fatty acids plasma TAG concentrations (Deckelbaum et al, 2006). Activation of PPARα enhances fatty acid metabolism, transport and β-oxidation, and suppresses TAG synthesis (Davidson, 2006). Both EPA and DHA induce acyl-CoA oxidase gene expression in a PPARα dependent manner in rat hepatocytes, (Harris et al, 2008) and suppress phosphatidic acid phosphohydrolase (PAP) and acyl-CoA: diacylglycerol hydrolase, which are key enzymes in TAG biosynthesis (Harris & Bulchandani, 2006). Fatty acids are weaker ligands for PPARγ (Xu et al, 1999). In human adipocytes, EPA and DHA increase lipoprotein lipase (LPL) expression possibly as a consequence of PPARγ induction (Khan et al, 2002). A study on mice has suggested that DHA, but not EPA may increase circulating adiponectin

(Vemuri et al, 2007). However, the effect of DHA *versus*. EPA on adiponectin concentrations in human subjects is uncertain. The following section reviews the role of adiponectin.

1.3 Adiponectin

Adiponectin belongs structurally to the collagen superfamily and is composed of an NH2-terminal collagen region and a COOH-terminal C1q-like globular domain (Radjainia et al, 2008). In the circulation, adiponectin is present in three oligomeric complexes, which have been shown to act though distinct signalling pathways and therefore have different biological functions (**Figure 1.3**). The trimeric adiponectin, the basic building block of adiponectin is also called the low molecular weight adiponectin (LMW) isoform (Oh et al, 2007; Tsao et al, 2003). The hexametric adiponectin (medium- molecular-mass) isoform is formed through the disulfide bond-mediated self-association of two homotrimers. The high molecular weight adiponectin (HMW) is the critical form for the biological activity of adiponectin (Oh et al, 2007).

As reviewed by Wang et al. (2008), both *in vitro* and animal- based studies support the role of the HMW as the major active form in mediating the metabolic action of adiponectin. It has been established that the HMW adiponectin possesses anti-diabetic and anti-atherogenic functions in humans and mice (Kobayashi et al, 2004). It has been found that coronary artery disease (CAD) is associated with a reduction in the HMW isoform, with either no change or an increase in the hexamer and trimer forms (Kobayashi et al, 2004). In 3T3-L1 adipocytes activation of AMPK significantly increases the concentrations of HMW adiponectin (Li et al, 2011). Animal studies showed that the HMW/total adiponectin correlated closely to hepatic insulin sensitivity (Pajvani et al, 2004). Significant weight reduction was also associated with an increase in HMW adiponectin, with no changes to the hexamer and trimer forms (Kobayashi et al, 2004). In recent studies, HMW adiponectin was associated with TAG/HDL-C, TC/HDL-C, LDL-C/HDL-C (Kawamoto et al, 2011), and administering thiazolidinediones (TZDs) to diabetic patients increased the serum HMW adiponectin , improved glucose and lipid profiles and blood pressure (Hirose et al, 2010).

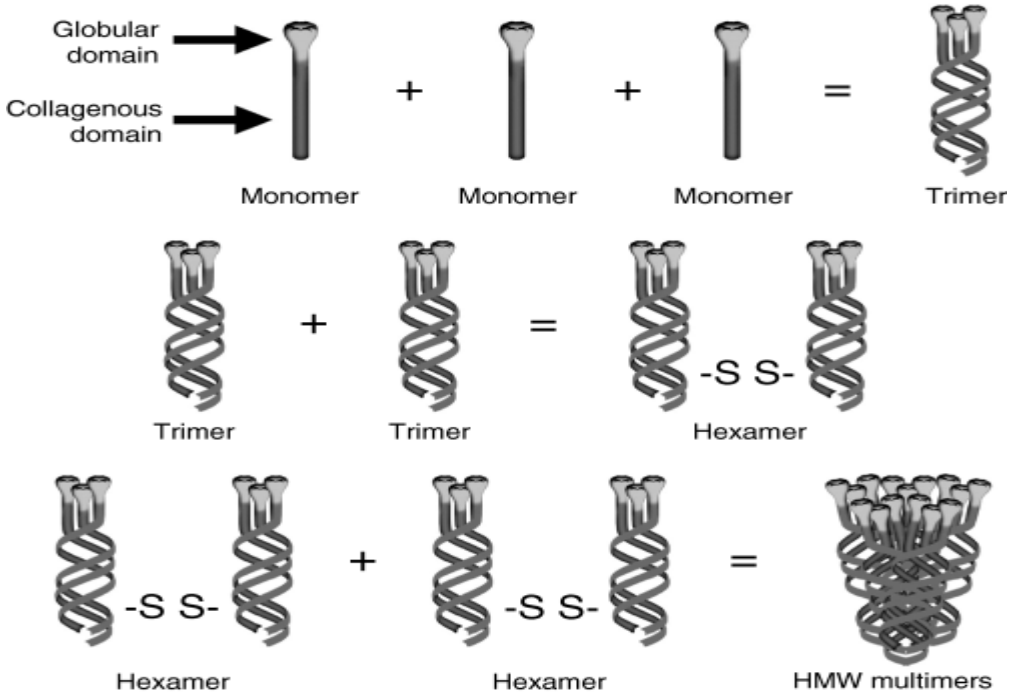

Figure1.3 Schematic model of adiponectin multimerisation. From (Oh et al, 2007)

1.3.1 Adiponectin and gender and age

The sexual differences in adiponectin concentrations has been well reported in humans, animals and cultured cells, with males having significantly lower plasma concentrations than females (Wang et al, 2008). In male mice, adiponectin concentrations increase 4-fold during sexual maturation compared to a 10-fold increase in females (Combs et al, 2003). The gender differences in plasma concentrations have been attributed primarily to the inhibitory effect of testosterone on adiponectin production (Liu & Liu, 2010; Wang et al, 2008). In cultured 3T3-L1 adipocytes, testosterone suppresses the secretion of adiponectin into the culture media (Nishizawa et al, 2002). Some studies have suggested that testosterone maybe involved in the post-transcriptional regulation of adiponectin (Combs et al, 2003; Nishizawa et al, 2002). Adiponectin concentrations generally increase with age, which may be explained by the changes of sex hormones associated with aging (Isobe et al, 2005; Adamczak et al, 2005). As insulin sensitivity declines with age, the increase in adiponectin associated with aging may reflect development of resistance or survival in those with higher adiponectin concentrations

1.3.2 Adiponectin receptors

Adiponectin is involved in the regulation of energy homeostasis and glucose and lipid metabolism through acting on two receptors; adiponectin receptor 1 (AdipoR1) and adiponectin receptor 2 (AdipoR2). AdipoR1 is the receptor for global and full length adiponectin, and almost all is expressed in skeletal muscle. However, AdipoR2 is more abundant in the liver and is the receptor for full length adiponectin (Kadowaki & Yamauchi, 2005).

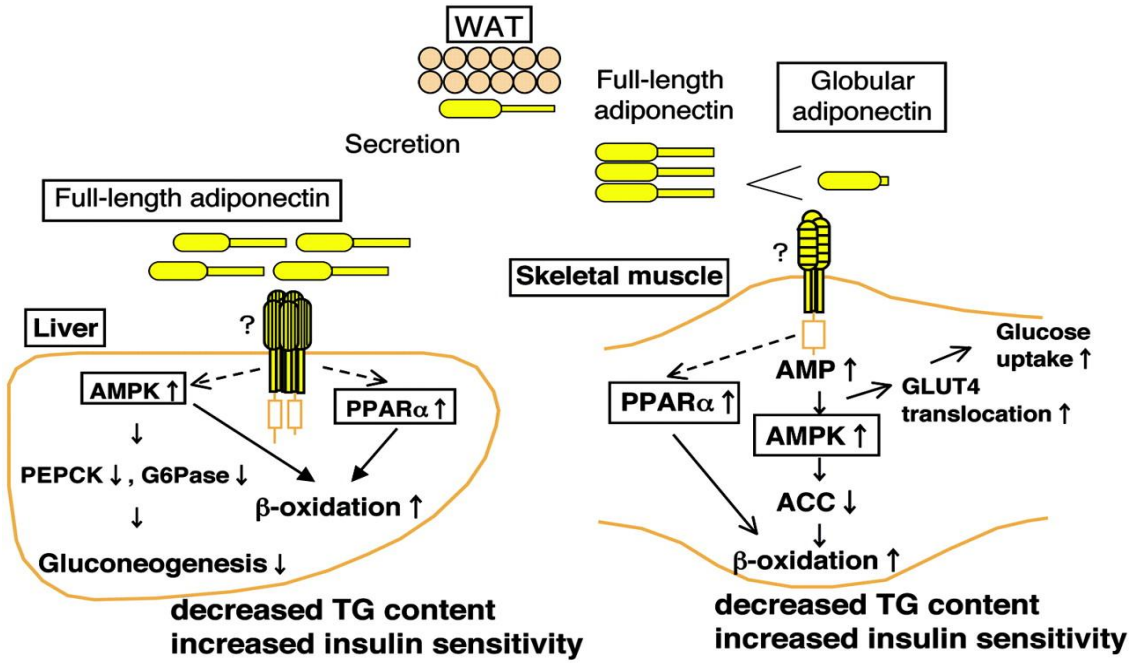

Figure1.4 Adiponectin mechanism of action. From (Kadowaki & Yamauchi, 2005)

Adiponectin can activate AMP-activated protein kinase (AMPK), which is a key energy sensor that maintains cellular energy homeostasis and PPARα in the liver and skeletal muscle (**Figure 1.4**). In the liver, full fragment adiponectin activates AMPK and PPARα via the AdipoR2. AMPK is activated by an increase in the intracellular AMP/adenosine triphosphate (ATP) ratio. This down-regulates the enzymes involved in gluconeogenesis including phosphoenolpyruvate carboxykinase (PEPCK) and glucose-6-phosphatase (G6Pase). It also increases the inhibitory phosphorylation of ACC, promoting fatty acid oxidation, and inhibits the action of other genes such as SREBP-1 required for fatty acid synthesis. The activation of PPARα by AMPK decreases the TAG content in the liver by stimulating fatty acid oxidation (Kadowaki & Yamauchi, 2005). In muscle, both full length and globular adiponectin act via the AdipoR1 receptor to stimulate fatty acid oxidation and glucose utilisation. AdipoR1 targets genes such as

CD36 involved in fatty acid transport, acyl coenzyme A oxidase (ACOD), which is involved in fatty acid oxidation, and uncoupling protein-2 (UCP-2) which is involved in energy dissipation as heat (Kadowaki & Yamauchi, 2005).

Therefore, as adiponectin increases fatty acid oxidation in the liver and muscles, it leads to a reduction in adipose tissue content and a decrease in pro-inflammatory cytokines, resulting in insulin signalling no longer being compromised. Insulin not only stimulates the production of adiponectin, but in turn, adiponectin sensitises tissues to insulin. The following section reviews the metabolic effect of adiponectin.

1.3.3 Adiponectin metabolic effects

1.3.3.1 Insulin sensitivity

Low adiponectin concentrations are associated with insulin resistance and obesity (Whitehead et al, 2006). Insulin resistance may explain its association with low adiponectin concentrations, or insulin resistance could be the consequence of a lack of adiponectin. The reduction of adiponectin concentration associated with insulin resistance could be caused by the disruption of the phosphatidylinositol 3-kinase (PI3K) insulin signalling pathway (**Figure 1.5**), as insulin stimulates adiponectin expression (Pereira & Draznin, 2005)

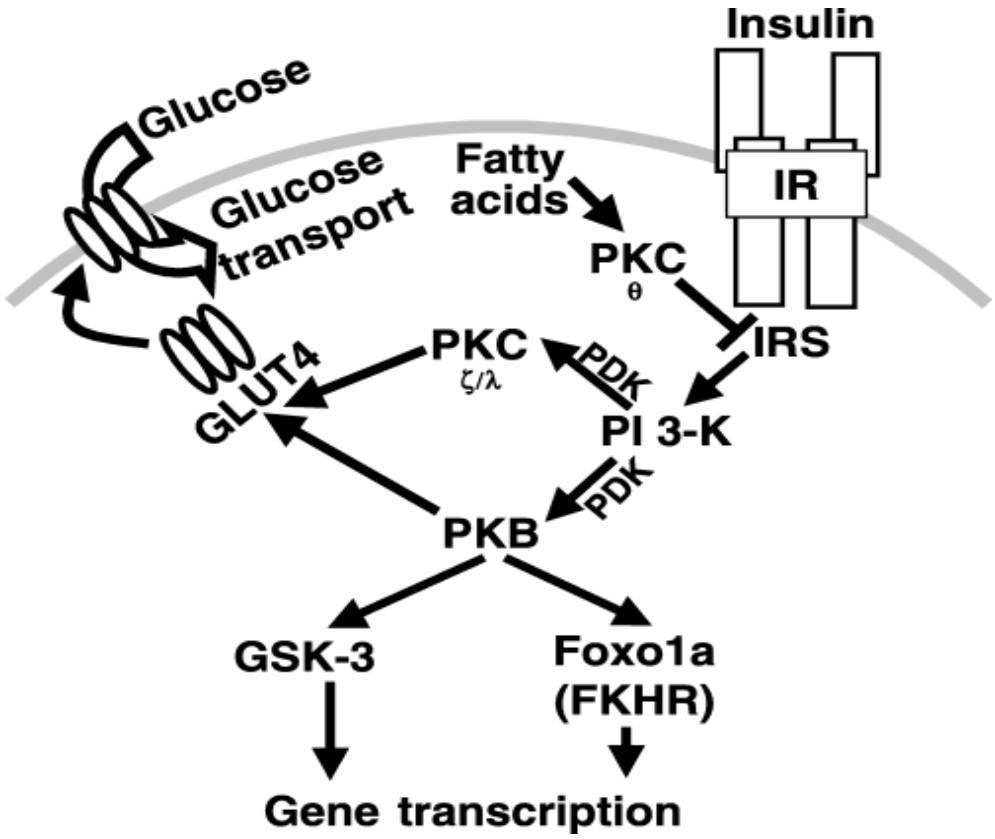

Figure1.5 Schematic representation of insulin signalling cascade. Insulin binds to the insulin receptors (IR) that activate PI3K through IRS. PI3K activates phosphoinositide-dependent Kinase (PDK) that will then mediate the activation of both protein kinase C (PKC) and protein kinase B (PKB). PKC can then activate and translocate glucose transporter type 4 (GLUT4) which regulates glucose uptake to the plasma membrane. PKB can also regulate the latest pathway and the transcription of target genes via glycogen synthase kinase 3 (GSK-3) or Forkhead box protein 01A (Fox01a). From (Schinner et al, 2005)

Pereira & Draznin (2005) have investigated the effects of insulin on adiponectin production. In this study 3T3-L1 mouse adipocytes were treated with increasing concentration of insulin. The insulin signalling pathways involved in the regulation of adiponectin production were examined by the inhibitors of PI3K and mitogen-activated protein kinase (MAPK) pathways. The study revealed that insulin stimulates adiponectin secretion in a dose-dependent manner and proceeds via the PI3K-dependant signalling pathway, but not via the MAPK pathway. Therefore, low adiponectin concentrations in insulin resistance may be explained by the blockage of the PI3K dependant pathway observed in insulin resistant states. This is supported by the work of Bogan & Lodish (1999), which showed that insulin-stimulated enhancement of adiponectin secretion, was blocked by pharmacological PI3K inhibitors. Foxo1 is a member of the forkhead transcription factor family and is involved in adipocyte

differentiation (Liu &Liu, 2010). Studies have shown that Foxo1-C/enhance binding protein (EBP) transcription complex which is enhanced by SIRT1, is critical in controlling adiponectin promoter activation and gene expression (Qiao & Shoa, 2006).

Alternatively, increased adiponectin secretion could be the normal physiological response to improved insulin sensitivity with increasing adiposity. Adiponectin treatment improved insulin resistance in lipotrophic mice (Yamauchi et al, 2001) and enhanced insulin sensitivity in male Wistar rats (Satoh et al, 2005). A recent meta-analysis of human studies showed that higher adiponectin concentrations are linked to a lower risk of developing T2D (Li et al, 2009).

Interactions between adiponectin and AdipoR1 increase the activation of PPARα, AMPK and p38 MAPK (Yamauchi et al, 2003). APPL1 is recognised as a key player in the regulation of insulin sensitivity through the activation of the adiponectin signal cascade, and acts as a positive regulator of adiponectin signalling (Deepa & Dong, 2009). Studies have shown that over-expression of APPL1 increases the phosphorylation of AMPK and p38 MAPK by binding to AdipoR1 (Mao et al, 2006). *In vitro*, Wang et al. (2007) provided a molecular mechanism by which adiponectin functions as an insulin sensitizer. They reported that adiponectin activates AMPK pathways by binding to its membrane receptor AdipoR1 and AdipoR2. This leads to the recruitment of the adaptor protein APPL1 and the activation of AMPK signally pathway. Activation of AMPK signalling reduces serine phosphorylation of IRS protein leading to enhanced IRS tyrosine phosphorylation and insulin signalling (Wang et al, 2007; Nishida et al, 2007). Adiponectin may also attenuate tumur necrosis factor-α (TNF-α) expression and inflammation in adipose tissue, therefore improving overall insulin sensitivity (Maury & Brichard, 2010). The following section describes the role of adiponectin and inflammatory markers associated with obesity.

1.3.3.2 Obesity

Obesity is associated with a reduction in serum adiponectin in many, but not all, subjects; this phenomenon has been partly attributed to the influence of inflammatory cytokines (Behre, 2007). Obesity, particularly which associated with T2D, is also characterised by an increased production of a wide range of inflammatory molecules, TNFα and interleukin-6 (IL-6) (Ouchi & Walsh, 2007). Butner et al. (2010) have suggested that adiponectin is a key mediator of inflammation in adipose tissue.

Attenuation of insulin signalling by pro-inflammatory cytokines would reduce the stimulation of adiponectin expression by insulin, leading to low concentrations of adiponectin associated with obesity and insulin resistance. Two transcription factor-signalling pathways have been linked to the pro-inflammatory effects of insulin resistance and obesity; c-Jun NH2-terminal kinase (JNK) and nuclear factor- κB (NF-κB). These pathways are activated by cytokonase such as TNF-α (Shoelson, 2007). TNF-α induced insulin resistance induces the expression of protein tyrosine phosphatase (PTP)-1B and mediates serine phosphorylation of the insulin receptor substrate-1 (IRS-1), which inhibits normal tyrosine phosphoration of IRS-1 and down regulates insulin signalling (Chuang et al, 2011), and inhibits insulin stimulated GLUT4 translocation (Nohara et al, 2011). Furthermore, this has been shown to phosphorylate PPARγ and decrease its deoxyribonucleic acid (DNA) binding activity (Liu & Liu, 2010). Adiponectin suppresses TNF-α induced IkBα-NFKB activation through a cAMP–dependant pathway (Ouchi et al, 2000). Mitochondrial dysfunction activates ER stress and JNK in adipose tissue, which may also explain decreased plasma adiponectin concentrations associated with obesity (Koh et al, 2007).

Elevated plasma NEFA, which occurs when body fat is mobilised, produces a defect in insulin-stimulated glucose transport and/or phosphorylation caused by a defect in insulin signalling (Boden & Chen, 1995). There is no simple relationship between the size of body fat stores and plasma NEFA concentrations. There is substantial variation between individuals with similar fat stores in their capacity to release NEFA and currently there is a limited understanding of the mechanisms that lead to the regulation of adipose tissue lipases. Epinephrine, glucagon and adrenocorticotrophic hormones stimulate lipolysis whereas insulin appears to suppress hormone sensitive lipase by changing is phosphorylation status (Frayn, 2003). Elevated plasma NEFA concentration

will lead to the accumulation of acetyl CoA and diacylglycerol, and the activation of PKC in liver, skeletal muscle and vascular endothelial cells. This interrupts insulin signalling by serine phosphorylation of IRS, resulting in a decrease in tyrosine phosphorylation of IRS (Boden, 2008). NEFA also inhibits the GLUT4 translocation to the cell membrane, thus disrupting insulin-mediated glucose uptake into skeletal muscle. In the liver, NEFA inhibits the insulin-induced suppression of glycogenolysis and gluconeogenesis (Gallagher et al, 2010). Therefore, attenuation of insulin signalling by elevated NEFA, inflammatory cytokines and mitochondrial dysfunction may contribute to the reduction in adiponectin concentrations associated with obesity and insulin resistance.

A 5-year follow-up study concluded that changes in body weight are inversely correlated with changes in adiponectin concentration (Arawaka et al, 2006). Treatment of obesity by either calorie restriction or bariatric surgery increases adiponectin concentrations (Yang et al 2001; Appachi et al, 2011). Marked weight-loss induced decrease in TNF-α (Dandona et al, 1998), improved insulin sensitivity (Agueda et al, 2010) and induced adiponectin gene expression (Coughlin et al, 2007). The following section reviews the association between serum adiponectin concentrations and lipid profile.

1.3.3.3 Plasma lipid profile

It has been reported that adiponectin is negatively correlated with TAG concentrations (Hotta et al, 2000) and positively correlated to HDL-C and Apo-A1 (Matsubara et al, 2002). The mechanism underlying the association between plasma adiponectin and dyslipidaemia may be a direct effect of adiponectin on inhibiting hormone sensitive lipase rather than an indirect caused by insulin resistance. Insulin is a well-known stimulator of adipose tissue LPL activity but is an inhibitor of hormone sensitive lipase (Schittmaye & Birner-Gruenberger, 2009). LPL plays a role in lipid metabolism and the storage of fat by catalysing the rate-limiting step in the hydrolysis of the TAG component present in circulating VLDL and chylomicrons. This reaction provides NEFA and 2-monoacylglycerol for tissue utilisation. Decreased adipose tissue LPL activity is seen in insulin resistant subjects (Kern, 1997). However, the hypertriglyceridaemia associated with metabolic syndrome is due to overproduction of VLDL TAG rather than impaired LPL activity, although in some cases i.e. type V

hyperlipoproteinaemia, impaired lipolysis is present (Thompson, 2004). The main driver for TAG synthesis in the liver is the flux of NEFA from adipose tissue which is a consequence of a failure of adipose tissue to suppress lipolysis. The glitazone class of drugs which are PPARγ agonists suppress the outflow of NEFA from adipose tissue and lower plasma TAG (Belfort et al, 2006). This suggests that adiponectin may have a role in regulating adipose tissue lipase.

1.4 *ADIPOQ* gene

Adiponectin is encoded by the *ADIPOQ* gene which is located on chromosome 3q27. It encodes a protein with similarity to collagens X and VIII and complement factor C1q. This gene is exclusively expressed in adipose tissue. It contains 2 introns and 3 exons within the region of 17 kb. It is estimated that genetic factors account for 30 to 70% of the variability of serum adiponectin concentrations (Menzaghi et al, 2002). The translation start point is located in exon 2. The promoter region from −676 to +41 has found to be sufficient for basal transcriptional activity, in which there are two elements; SREBP and CCAAT/enhancer binding (Gu, 2009), as shown in **Figure 1.6.**

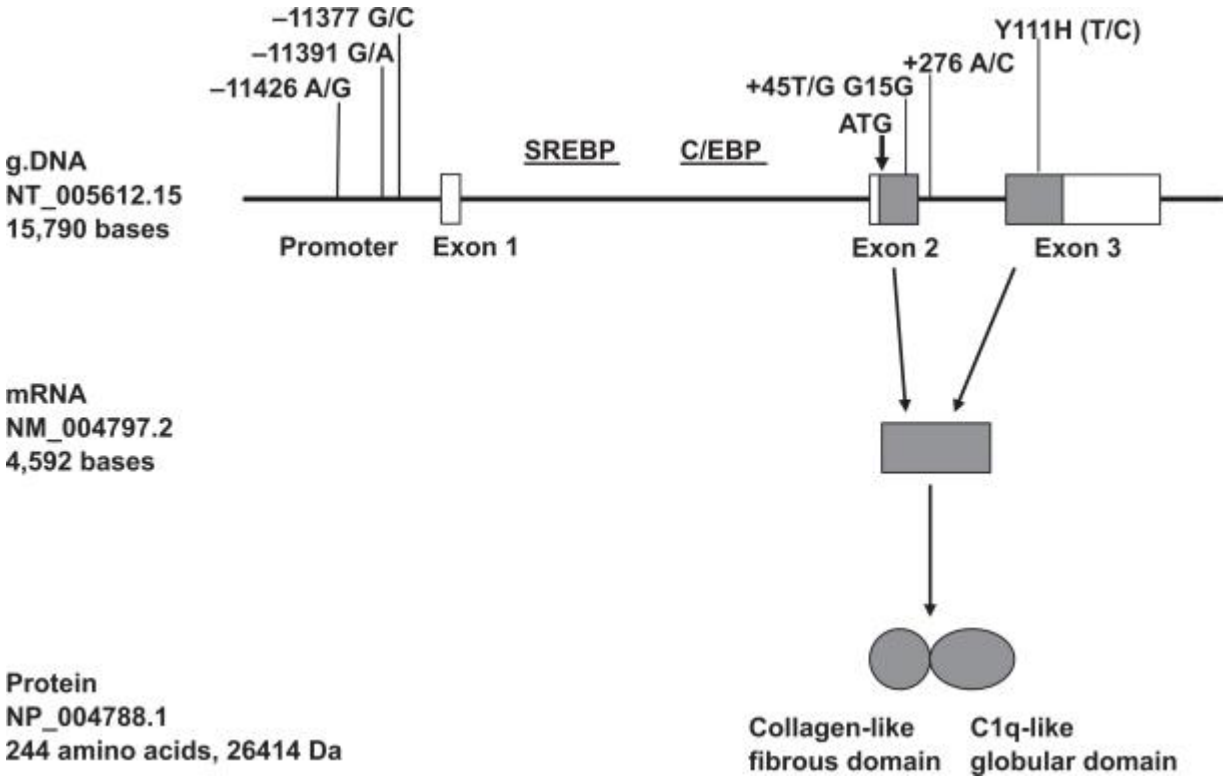

Figure1.6 Genomic DNA, mRNA and protein of adiponectin. From (Gu, 2009)

1.4.1 *ADIPOQ* polymorphisms

The *ADIPOQ* gene is very polymorphic; several single nucleotide polymorphisms (SNPs) have been detected. From the known polymorphisms in the National Centre for Biotechnology Information (NCBI) and in the literature, 53 SNPs of *ADIPOQ* have been identified (Heid et al, 2006). Some of these polymorphisms have been associated with the expression of adiponectin, suggesting their determination of circulating concentrations (Yang & Chuang, 2006). Associations were found between +276 G-allele and lower adiponectin concentrations in the Spanish population, healthy Caucasians, nondiabetic Koreans and obese Japanese subjects (Gonzalez-Sanchez et al, 2005; Mackevics et al 2006; Jang et al, 2005; Hara et al, 2002). An association was also reported in healthy young Finnish subjects where the +276 T-allele was associated with elevated adiponectin concentrations (Mousavinasab et al, 2006).

Woo et al. (2006) showed a significant association between SNP alleles -11391A, +267T and -10066G with higher adiponectin concentrations in White Americans. A similar association with -11391 A and +267 T-alleles was found in African Americans. They also showed that three SNP haplotypes +45, +276 and +349 T/T/A and T/G/G were significantly associated with higher adiponectin in White American, but not Africans. In the same study the strongest association with serum adiponectin was found with haplotypes from the promoter through to the second intron SNPs -11391 to +349 in White Americans and SNPs -11391 to + 45 in African Americans (Woo et al, 2006). In a more recent study, SNP association with adiponectin was tested in two independent populations of Caucasian females. In the Chingford cohort (n = 808), the common allele of -10066 G/A and the rare alleles of 11391 G/A, -7734 C/A, +276 G/T and +3228 C/T were significantly associated with higher adiponectin concentrations. In the Twins UK cohort (n = 2718), the same association was found with all SNPs except for +3228 C/T (Kyriakou et al, 2008). In the CARDIA study, -10066 G/A, -11391 G/A and +276 G/T were strongly associated with serum adiponectin concentrations in White subjects, with dose – response relationship of mean adiponectin concentrations across genotypes. This study also reported that only -11391 G/A was significantly associated with serum adiponectin in African Americans (Wassel et al, 2010) similar association was reported by (Heid et al 2006; Vasseur et al, 2002).

Association between *ADIPOQ* gene variants with metabolic syndrome risk factors has been established in many studies. Jang et al. (2005) for example, reported that carriage of the +276 G-allele could be a significant contributor to higher CVD risk in Koreans. This result seems to contradict an Italian study which reported an increased risk of CAD in the carriers of the +276 T-allele (Filippi et al, 2004). More recent studies suggested that the +276 T-allele appeared to protect from myocardial infarction and T2D (Chiodini et al, 2010) and is associated with a decreased risk of CAD in T2D patients (Esteghamati et al, 2011) and +276 G-allele with the presence of hypertension (Leu et al, 2011).

Some polymorphisms in the *ADIPOQ* gene have been shown to be associated with some risk factors, such as insulin resistance and glucose intolerance. In a Spanish cross sectional study, the G276G genotype was associated with increased risk of impaired glucose tolerance (Gonzalez-Sanchez et al, 2005). In a cross sectional study of 413 healthy Caucasians, +276 T-allele was significantly associated with a lower homeostatic model assessment of insulin resistance (HOMA-IR) (Menzaghi et al, 2002). However, an adverse effect of +276 T-allele on insulin resistance was found in non-diabetic Greek women, who exhibited higher fasting insulin levels and thus higher HOMA-IR (Melistas et al, 2009). The +276 G-allele was significantly associated with higher HOMA-IR in Koreans (Jang et al, 2005), non-diabetic Italian subjects (Menzaghi et al, 2002) and Japanese subjects (Hara et al, 2002). Others reported no association between *ADIPOQ* variants and insulin resistance indexes (Vasseur et al, 2002). Some other associations with HDL-C, LDL-C and diastolic BP were also reported. Carriers of the +276 T-allele have been associated with LDL-C and lower HDL-C concentrations (Berthier et al, 2005), elevated diastolic BP (Mousavinasab et al, 2006) and lower systolic BP (Menzaghi et al, 2002). Carriers of +276 G-allele were found to have significantly higher concentration of TAG (Jang et al, 2005).

Association studies have also demonstrated significant interaction between *ADIPOQ* gene variants and quantitative measurements of obesity. Significant but inconsistent associations were found with BMI: the +276 G-allele was associated with higher BMI in Italian subjects (Menzaghi et al, 2002) but with lower BMI in Swedish women and African Americans (Ukkola et al, 2003; Beebe-Dimmer et al, 2010). A recent meta-analysis, suggested that the *ADIPOQ* G276T is associated with obesity susceptibility

(Yu et al, 2011). Higher WHR was found in carriers of the -11391 A-allele (Dolley et al, 2008).

The inconsistencies in the results may be attributed partly to differences of the racial composition of the populations; differences in the frequencies of SNPs–between races could be due to different patterns of Linkage disequilibrium (LD) (Wassel et al, 2010). The presence of co-morbidities in the study populations could largely explain such controversy. Age and environmental factors (food intake, fatty acid composition of the diet), may differ significantly between study populations, thus leading to this inconsistency (Ntalla et al, 2009). Therefore, the following section reviews the effect of diet on adiponectin concentrations.

1.4.2 Adiponectin, *ADIPOQ* polymorphisms and diet

1.4.2.1 Adiponectin concentration and diet

A study on Japanese men has reported that adiponectin concentrations are independently related to dietary factors, smoking and physical activity (Tsukinoki et al, 2005). Diets low in glycaemic load and high in fibre have been significantly associated with high adiponectin concentrations in diabetic men (Qi et al, 2005) and healthy women (Yannakoulia et al, 2008). Pischon et al. (2005) also reported that high glycaemic load is associated with lower adiponectin concentrations whereas moderate intake of alcohol was found to be associated with high adiponectin concentrations. Following a high saturated fat diet Van Dijk et al. (2009) reported that *ADIPOQ* expression and serum adiponectin concentrations were significantly decreased. Adherence to the Mediterranean diet for 2 years along with increasing physical activity decreased BMI and increased adiponectin concentrations in postmenopausal obese women (Esposito et al, 2003), diabetic women (Mantzoros et al, 2006) and in healthy adults (Fragopoulou et al, 2010). Women from the Nurse's Health Study, with the highest adherence to healthy eating patterns had 23% higher adiponectin concentrations than those with the lowest adherence (Fargnoli et al, 2008). Other reported a negative correlation between adiponectin concentrations and the consumption of trans fatty acids and a positive correlation with vegetable consumption (Cassidy et al, 2009).

EPA and DHA have been reported to increase plasma adiponectin concentrations through stimulation of *ADIPOQ* gene expression in adipocytesin mice (Flachs et al,

2006). Similarly, Todoric et al. (2006) reported that 6 weeks of omega-3 supplementation significantly increased adiponectin concentrations in diabetics and non-diabetics rats. Human studies have shown that 24 weeks supplementation of a low-fat/high carbohydrate diet with EPA + DHA in overweight insulin-resistant women resulted in beneficial effects on improved risk factors associated with CVD and improved adiponectin concentrations (Krebs et al, 2006). Many animal studies have demonstrated that EPA and DHA intake result in a significant increase in adiponectin concentrations (Hassanali et al, 2010; Kuda et al 2009; Duda et al, 2009; Higuchi et al, 2008). EPA alone increases adiponectin concentrations in patients with coronary stenoses (Rasmussen et al, 2009) and in obese Japanese (Satoh et al, 2009).

In vitro, the independent action of EPA and DHA in 3T3-L1 adipocytes was investigated. Both EPA and DHA increased adiponectin secretion, however, this increase was 40% more with DHA treatment compared to EPA. Only DHA enhanced PPARγ and adiponectin mRNA expression compared with the control (Oster et al, 2010). The same group later reported that the effect of EPA on adiponectin does not appear to be entirely PPARγ mediated. In their study, they cultured human adipocytes with DHA or EPA. Both these significantly increased adiponectin concentrations by 88 % and 47 %, respectively. However, PPARγ antagonism fully abolishes the DHA-effect on increased adiponectin secretion, but only partially attenuated the EPA-mediated response (Tishinsky et al, 2011). Furthermore, DHA but not EPA may reduce insulin resistance in mice, which may be mediated through an increase in circulating adiponectin through PPARγ activation (Vemuri et al, 2007). As far as could be ascertained, no studies have compared the effects of supplementation with EPA *versus.* DHA on adiponectin concentrations

1.4.2.2 *ADIPOQ* polymorphisms and diet

Some studies have explored the influence of *ADIPOQ* gene polymorphisms on serum adiponectin in response to dietary factors. In a population of healthy Greek children, the +276 G/T x fibre interaction was significantly associated with adiponectin concentrations. The +276 G/G homozygotes subjects had significantly higher adiponectin concentrations compared to +276 T-allele carriers under conditions of low fibre intake (Ntalla et al, 2009). Chang et al. (2009) determined the effect of +276 G/T on adiponectin concentrations and HOMA-IR after 12 weeks intervention (replacement

of refined rice with whole grains, a high intake of vegetables and regular exercise) in overweight-obese patients with impaired fasting glucose or newly diagnosed T2D. After a 12-week trial, both serum glucose and HOMA-IR were reduced in G-allele carriers of +276, however, no differences were found in adiponectin concentration.

A study in US Whites, demonstrated that in the highest 50th percentile of MUFA intake, carriers of the minor allele at the *ADIPOQ* -11391 G/A had a lower BMI and a 48% reduction in the risk of obesity compared with G/G homozygotes. No interaction was found in the lowest 50th percentile of MUFA intake (Warodomwichit et al, 2009). Perez-Martinez et al. (2008) found on a high MUFA diet, the C/C homozygous men for the -11377 were significantly less insulin resistant, compared to a high SFA diet. No dietary interactions were found with the -11391 G/A, +45 T/G and +276 G/T variants in determining other variables. In a recent study, an interaction between *ADIPOQ* -11377 C/G genotype with SFA, but not MUFA or PUFA, significantly affected HOMA-IR. However, there were no significant effects on serum adiponectin concentration (Ferguson et al, 2010). There is still a limited number of studies looking at the diet-gene interaction with *ADIPOQ* variants. The *ADIPOQ* expression is induced by PPARγ activation. Consequently, the following section reviews the PPARγ role and *PPARG* gene.

1.5 Peroxisome proliferator-activated receptor γ

The peroxisome-proliferator activated receptors (PPARs) are transcriptional regulators of cellular differentiation, development and lipid metabolism, activated by unsaturated fatty acids (Walczak & Tontonoz, 2002). One of the most studied isoforms of the PPAR family is PPARγ. It is a transcription factor that is a member of the nuclear hormone receptor superfamily (Lehrke & Lazar, 2005), which has been of particular interest because of its dominant role in the control of the expression of genes related to inflammation, adipose cell differentiation, atherosclerosis, metabolism and cancer (Costa et al, 2010).

PPARγ controls biological functions by controlling the expression of specific genes, mainly in a ligand-dependant manner (Lehrke & Lazar, 2005). Ligands occur either naturally or in synthetic form. Natural ligands include linolenic, EPA, DHA and AA, as well as prostaglandin (Xu et al, 1999). The synthetic ligands include TZD, non-steroid and anti-inflammatory drugs (Lehrke & Lazar, 2005).

1.5.1 Role in adipogenesis

PPARγ2 regulates adipocytes differentiation and may serve to link the process of adipocytes differentiation to systemic lipid metabolism (Tontonoz & Spiegelman, 1994). PPARγ2 deficient mice have exhibited a reduction in white adipose tissue (WAT), decreased expression of adipogenic genes and lower lipid accumulation in adipose tissue, while insulin sensitivity has also been found to be dramatically impaired (Zhang et al, 2004). Meanwhile, the expression of IRS-1 in PPARγ2 deficient mice was significantly reduced in WAT, liver and skeletal muscle. Moreover, in these mice, the expression level of GLUT4 in skeletal muscle was also dramatically decreased (Zhang et al, 2004).

As reviewed by Lowell (1999). One of the adipocyte-specific genes is adipocyte-specific fatty acid–binding protein gene (*ap2*), which has binding sites for the two major adipogenic transcription factors: CCAAT/enhancer binding protein-α (C/EBPα) and PPARγ. During the early stages of adipocytes differentiation, C/EBPβ and C/EBPδ are induced and thought to in turn induce expression of PPARγ and C/EBPα, culminating in the terminal differentiation of adipocytes (Lowell, 1999; Walczak & Tontonoz, 2002). PPARγ activity will further increase through ligands such as PUFA. This will further

induce C/EBPα and also increase its own expression (Lowell, 1999). Another transcription factor associated with adipocyte differentiation and cholesterol homeostasis is adipocyte differentiation and determination factor 1/SREBP-1 (ADD-1/SREBP-1), found to induce PPARγ mRNA levels in 3T3 –L1 and HepG2 cells and therefore regulate different pathways of lipid metabolism (Fajas et al, 1999).

1.5.2 Role in insulin sensitivity

Activation of PPARγ increases GLUT1 and GLUT4 expression and lowers NEFA levels. It also enhances insulin signalling pathway by upregulating protein levels of IRS (Smith, 2001) and increasing the expression of c-Cbl-associated protein (CAP) which is a signalling protein that may be involved in the insulin-stimulated tyrosine phosphorylation of c-Cbl (Ribon et al, 1998; Gurnell, 2003). Incubating human adipocytes with rosiglitazone significantly increased the p85 subunit of PI3K, which is a major component of insulin action and UCP-2 mRNA levels and enhanced GLUT4 expression (Rieusset et al, 1999; Gurnell, 2003). On the other hand, treating 3T3-L1 cells with artepillin C, a PPARγ ligand had no effect on the insulin signalling cascade, but enhanced the expression and plasma membrane translocation of GLUT1 and GLUT4 and adipocyte differentiation (Choi et al, 2011).

1.5.3 Role in lipid homeostasis

PPARγ can modulate lipid homeostasis through the regulation of genes, including acyl:cholesterol acyl transferase (*ACAT*), *LPL*, phospholipiase A (*PLA*), *aP2*, and the fatty acid transport proteins *(FATP*) and *CD36* (Walczak & Tontonoz, 2002). The TZD class of drugs, which are PPARγ agonists, decrease plasma NEFA and TAG and increasing HDL-C (Boyle et al, 2002; Belfort et al, 2006, Komatsu & Node. 2010) suggesting that the primary mechanism of action is a decreased outflow of NEFA from adipose tissue.

1.6 *PPARG* gene

The human *PPARG* gene is located on chromosome 3 at position 3p25 (Ackert-Bicknell& Rosen, 2006). The *PPARG* gene includes 9 exons and exceeds 100 kb in length. It measures 146,483 base pairs in length, translating into 476 amino acids. Four different subtypes of PPARγ mRNA (-γ1,-γ2,-γ3 and -γ4) transcribed from four

different promoters have been identified, which give rise to two different PPARγ proteins (Zieleniak et al, 2008) as shown in **Figure 1.7**. The transcription from promoter – γ1, - γ3 and- γ4 mRNA results in a PPAR- γ1 protein of 477 amino acids. The PPAR- γ2 protein of 505 amino acids is produced by transcription from promoter γ2 (Zieleniak et al, 2008), exclusively expressed in adipose tissue (Lehrke & Lazar, 2005).

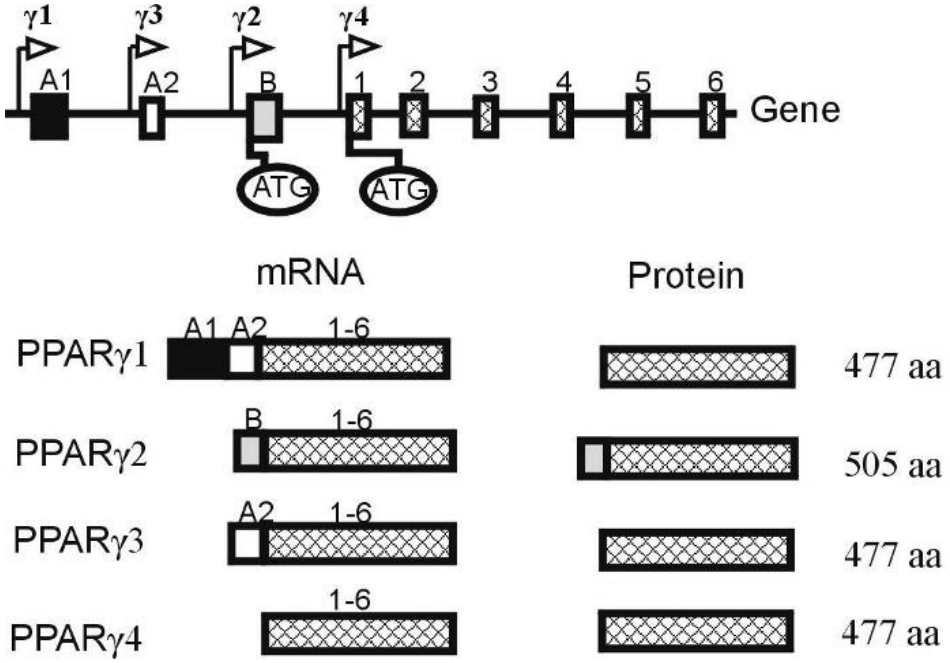

Figure1.7 Scheme of the genomic structure of the human *PPARG* gene and the structures of the PPARγ protein isoforms. From (Zieleniak et al, 2008)

1.6.1 *PPARG* Pro12Ala polymorphism

Since PPARγ is involved in the regulation of several genes in many tissues, genetic variation of the *PPARG* gene is likely to be associated with an alteration of the expression levels of target genes (Costa et al, 2010). The most widely studied SNP amongst the *PPARG* genes is Pro12Ala, which occurs in the PPARγ2 isoform and is located in codon 12 of exon 3. It involves a C-to-G base substitution, resulting in a mis-sense mutation in which proline is replaced by alanine (Tellechea et al, 2009). This polymorphism was first identified in 1997, with rare allele frequencies of 12% in Caucasians, 4 % in Japanese and 1 % in Chinese (Stumvoll & Haring, 2002). The frequency of the minor allele is 0.076 in Europeans (http://www.ncbi.nlm.nih.gov/snp; build 132 accessed 1/09/2011).

Numerous studies have investigated the association of this polymorphism and the risk of obesity and diabetes; however, the results are still very controversial. A recent meta-analysis of 41 studies, including 30,612 subjects, reported a protective effect of the Ala12-allele against T2D. This study found a significant association between the Ala12-allele and the lowest risk of T2D, greater insulin sensitivity and lower serum insulin in overweight Caucasians, however no association was found in non-Caucasians (Huguenin & Rosa, 2010). Similarly, Radha et al. (2006) found that the Ala12-allele did not protect against T2D in South Asians but did protect Caucasians. Altshuler et al, (2000) reported a significant 1.25 fold increase in diabetes risk associated with the more common allele Pro12 in Caucasians and Asians. A protective effect of the Ala12-allele, lowering the risk of T2D, was also reported in non-obese Type 2 diabetic patients (Deeb et al, 1998) and in normoglycaemic and Type 2 diabetic subjects (Chistiakov et al, 2010; Ereqat et al, 2009; Meshkani et al, 2007). However, a meta-analysis of 57 cohorts in subjects with normal or impaired glucose tolerance suggested no significant effect of the Pro12Ala on diabetes-related traits across all studies; this meta-analysis reported a protective effect of the Ala12-allele carriers and insulin sensitivity only on selected subgroups, such as Caucasians and obese subjects (Tonjes et al, 2006).

Since PPARγ plays a key role in adipocyte differentiation, the influence of the Pro12Ala on obesity measures has been of major interest, despite being extremely subtle. In lean individuals, the Ala12-allele has been associated with reduced risk of obesity (Deeb et al, 1998) but with increased risk of weight gain in a large cohort of White obese British (Masud & Ye, 2003). This was supported by Tonjes's meta-analysis, where amongst Caucasians, Ala12-allele carriers had greater BMI (Tonjes et al, 2006). This association was also found in the Québec Family Study, where Ala12-allele carriers had higher BMI, waist circumference and fat mass (Robitaille et al, 2003). A recent meta-analysis suggested that the interaction is greatly influenced by ethnic background as Pro12Ala polymorphism frequency is known to vary with ethnicity, with Caucasians having a higher frequency of the Ala12-allele compared with various types of Asian population (Gouda et al, 2010). Other influences could be the interaction with environmental factors, such as diet and genetic background, i.e., the interaction effect of the Pro12Ala polymorphism with Trp64Arg polymorphism in the β3-adrenegic receptor, which has been reported to influence BMI and insulin concentrations (Hsueh et al, 2001).

Some studies have investigated the association between Pro12Ala and adiponectin concentrations. Yamamoto et al. (2002) reported that serum adiponectin concentrations were significantly increased in subjects with the Pro/Ala genotype compared with the Pro/Pro genotype in Japanese subjects. This association was also reported in Finnish men, in which the Ala12-allele variant was associated with elevated adiponectin concentrations (Mousavinasab et al, 2005). In contrast, Takata et al. (2004) found that Pro12Ala substitution is associated with reduced serum adiponectin concentrations in young Japanese men. There was no such association reported in healthy Europeans, Asian Indians, healthy women or women with polycystic ovary syndrome (Thamer et al, 2003; Radha et al, 2007; Orio et al, 2004).

A recent meta-analysis investigated the association of Pro12Ala polymorphism with TC, LDL-C, HDL-C and TAG (Huang et al, 2011). The results of this suggested that Ala12-allele carriers have significantly increased total cholesterol (TC) in males and increased HDL-C in healthy subjects. They also found that Ala/Ala homozygote Caucasians have lower blood TAG compared with Pro/Pro homozygotes, which supports findings in the Spanish population, where a protective effect of the Ala12-allele in lowering total TAG concentrations (Gonzalez Sanchez et al, 2002). Conflicting results were reported by Beamer et al. (1998), who found a protective effect of the Pro12-allele in lowering TAG and increasing HDL-C concentrations in male subjects. Similarly Hamada et al. (2007), reported significantly higher serum TAG concentrations and a large area in small dense LDL (sdLDL) in Ala12-allele carriers, compared with Pro/Pro homozygotes. Ereqat et al. (2009) and Mori et al. (2001) also reported an increase in TC in Ala12-allele carriers. Other studies have reported no association between Pro12Ala and plasma lipids concentrations (Ringel et al, 1999; Meshkani et al, 2007). In a recent case control study, Ala12-allele was associated with reduced risk of CAD in Italian population (Galgani et al, 2010)

The direct effect of this polymorphism was reported in functional studies, which have stated that Ala12-allele variants present a decreased binding affinity to peroxisome proliferator response element (PPRE) and thus reduced transactivation ability both in TZD-induced adipogenesis and a luciferase reporter gene assay (Masugi et al, 2000; Deeb et al, 1998). Both reports suggest that Ala variants may contribute to the variability in adipose tissue formation and insulin sensitivity in the general population. The following section will review the influence of dietary ligands on *PPARG* Pro12Ala.

1.6.2 *PPARG* Pro12Ala polymorphism and diet

As previously mentioned, PPARγ-2 is a ligand-dependant nuclear receptor, which promotes adipocyte differentiation and TAG storage. Fatty acids are known natural ligands of PPARγ. However, their affinity depends largely on their chain length and degree of saturation (Xu et al, 1999). Thus the metabolic impact of this polymorphism is potentially dependent on gene-environmental interactions.

Results from the Québec Family Study have indicated that total fat intake was positively associated with increased waist circumference in Pro/Pro homozygotes but not in Ala12-allele carriers (Robitaille et al, 2003). Similar observations were observed with BMI and total fat intake in Pro/Pro homozygotes but not in Ala12-allele carriers (Memisoglu et al, 2003). Luan et al. (2001), examined the interaction between polyunsaturated: saturated fat (P:S) and Pro12Ala polymorphism in 592 non-diabetic Caucasians. No significant association between either fasting insulin or BMI and the Pro12Ala polymorphism was found when dietary fat intake was not considered. However, when the P:S ratio was considered, a negative association was found between both fasting insulin and BMI and the P:S ratio in Ala12-allele carriers, but not in Pro/Pro homozygotes. Heikkinen et al. (2009) generated a knock-in mouse model for this variant. They demonstrated that on a chow diet, Ala/Ala mice are leaner, have improved plasma lipid profiles and insulin sensitivity, together with a longer life span than Pro/Pro mice. A three-month course of fish oil supplements (3.6g of n-3 LCP) significantly decreased serum TAG in healthy Ala12-allele carrier subjects but not in Pro/Pro homozygotes. This study found no difference in heparin plasma LPL activity following fish oil supplementation (Lindi et al, 2003).

The Pro12Ala polymorphism responses were compared in a three year longitudinal trial of 522 subjects with impaired glucose tolerance randomized to either an intensive diet (lowering intake of dietary fat) and exercise group or control group. In the intervention group, individuals with the Ala/Ala lost more weight than Pro/Pro (Lindi et al, 2002). In a more recent study, the effect of Pro12Ala polymorphism in relation to adiposity was investigated in 774 high risk subjects on a Mediterranean diet over two years. After two years, the Ala12-allele carriers in the control group had higher waist circumference. However, this adverse effect was reversed among Ala12-allele carriers allocated to the Mediterranean diet (Razquin et al, 2009). The same effect was reported by a prospective

cohort study of 3,075 older adults, investigating the association between Pro12Ala, food patterns and body composition. Food intake was assessed with a semi-quantitative food frequency questionnaire: participants following a healthy diet characterised by a higher intake of low-fat dairy products, whole grains, fruit, vegetables, poultry and fish have significantly lower levels of adiposity in male Ala12-allele carriers but this was not the case for Pro/Pro homozygotes (Anderson et al, 2010). These studies suggest that Ala12-allele carriers may show different responses to diet fat intake compared with Pro12-allele carriers.

1.6.3 *ADIPOQ* and PPARγ

PPARγ agonists such as TZD have been clearly shown to increase serum adiponectin concentrations in both human and rodents (Iwaki et al, 2003). Both natural ligands such as PUFAs and artificial ligands such as TZDs enhanced the expression of adiponectin mRNA in adipose tissue and dramatically increased the plasma concentration of adiponectin (Maeda et al, 2001). The mechanism involves a functional PPRE and a responsive element of liver receptor homolog-1 (LRH-1) both located in the human adiponectin promoter (Iwaki et al, 2003). *ADIPOQ* gene expression is induced by PPARγ ligands via the direct binding to PPARγ/RXR heterodimer to the PPRE in adiponectin promoter. LRH-1 also binds to LRH-R, enhancing the transactivation of adiponectin promoter, thus enhancing the production and secretion of adiponectin from adipocytes (Iwaki et al, 2003).

1.7 Peroxisome proliferator-activated receptor α

PPARα is another member of the group of nuclear receptors that regulate key proteins involved in inflammation, fatty acid oxidation, lipoprotein metabolism (Vallim & Salter, 2010), extracellular lipid metabolism and haemostasis (Yoon, 2009). It is mostly expressed in tissues with high levels of fatty acid oxidation, such as liver and muscle (Auboeuf et al, 1997), and it regulates target genes involved in the transportation and oxidation of fatty acids (Aoyama et al, 1998). The cytochrome P450 system, which is induced to metabolise foreign compounds including some lipids, is capable of producing PPARα ligands (Ng et al, 2007). PPARα ligands include exogenous lipid-lowering drugs such as clofibrate, fenofibrate and endogenous fatty acids, which include branched chain saturated (such as phytanic acid) and C_{20-22} unsaturated fatty acids (such as erucic acid 22:1n-9) (Yoon, 2009; Corton et al, 2000). In the case of erucic acid, peroxysomes are induced to enable the microsomal chain shortening of erucic acid to 18:1 so that it can form carnitine esters and be translocated into the mitochondria for beta-oxidation (Clouet et al, 1982).

Like PPARγ, ligand-activated PPARα heterodimerises with RXR-α before binding to target gene promoters (Zieleniak et al, 2008), which usually contain one or more PPREs (Chu et al, 1995). In addition, PPARα transactivation is modulated by cofactors or corepressors (Yoon, 2009), which in the absence of a ligand inhibit its activity (Pyper et al, 2010). Studies have also found that PPARα can modulate its own expression (Corton et al, 2000; Pyper et al, 2010; Pineda et al, 2002) and transcriptional activity is also regulated by phosphorylation, which stabilizes its binding to the PPRE (Yoon, 2009). Studies in mice have shown that PPARα deficiency results in late-onset obesity (Costet et al, 1998) and the disruption of lipid metabolism (Djouadi et al, 1998).

1.7.1 Role in lipid homeostasis

In the liver, fibrate agonists of PPARα enhance FATP and acyl coenzyme synthetase (ACS) which generates fatty acyl-CoA, carnitine palmitoyl transferase-1 (CPT-1) essential for facilitating the entry of fatty acyl carnitine into mitochondria, and genes involved in mitochondrial β-oxidation (Staels et al, 1998). Other genes involved in peroxisomal (Coleman et al, 2002, Jia et al, 2003) and microsomal β-oxidation (Reddy & Hashimoto, 2001) are tightly regulated by PPARα.

Clofibrate (an amphipathic carboxylic acid) was one of the first drugs to be shown to be a ligand for the PPARα receptor. PPARα activates cytochome P450 enzymes mainly in the liver that are able to adapt to oxidize fatty acids that are unable to be oxidized within the mitochondria. The cytochrome P450 enzymes clusters generated hydrogen peroxides and so were called peroxysomes (Wray et al, 2009). Prior to the discovery of PPAR receptors, clofibrate had been shown using radioisotope tracer studies in man to decrease hepatic TAG synthesis and VLDL secretion, thus lowering TC and TAG. Its use as a drug fell out of favour when it was shown to have toxic side effects on the liver. Other fibrate compounds have been developed that do not show this hepatotoxic effects and their lipid lowering effects have been reviewed by Shah et al. (2010). Some of these had effects on stimulating LPL and apoC-III expression (Lemieux et al, 2003). PPARα induces *LPL* gene transcription (Vallim & Salter, 2010; Staels et al, 1998) and represses the expression of apoC-III, a natural inhibitor of LPL activity (Lemieux et al, 2003), which further enhances LPL-mediated catabolism of VLDL production (Staels et al, 1998). PPARα activation decreases mRNA concentration of fatty acid synthase (*FASN*), resulting in reduced synthesis of TAG (Jia et al, 2011). Hogue et al. (2008) reports that fibrate increases triacylglycerol rich lipoprotein (TRL) Apo B-48 as well as VLDL Apo B-100 clearance in T2D mellitus men with marked hypertriglyceridemia.

The FIELD study showed that in T2D subjects, fibrate treatment enhanced the transcription of Apo A-1, leading to increased Apo A-1 production (Taskinen et al, 2009). Therefore, PPARα activation by fibrates and other ligands, evokes a normolipidaemic response by reducing TRL, LDL-C and VLDL production, increasing TAG lipolysis and enhancing HDL-C metabolism (Lefebvre et al, 2006).

1.8 *PPARA* gene

The human *PPARA* gene has been mapped to the chromosomal region 22q12-q13.1. It spans 83.7 kb and is composed of 8 exons. The *PPARA* gene is highly polymorphic and contains 15 coding SNPs (http://www.ncbi.nlm.nih.gov/snp; build 132 accessed 15/12/10). *PPARA1* and *PPARA2* are two isoforms of the *PPARA* gene and both are expressed in human tissue (Golembesky et al, 2008). *PPARA1* encodes the entire region, whereas *PPARA*2 is truncated at exon 6 and lacks at least 200 base pairs. This results in a frame shift introducing a premature stop codon 3' of the deletion (Golembesky et al, 2008; Gervois et al, 1999). This truncation leads to the absence of the DNA-binding domains and parts of the hinge region, which prevents activation by the ligand (Gervois et al, 1999). Therefore, all studies have investigated the *PPARA1* isoform because of its known protein activity (Golembesky et al, 2008).

1.8.1 *PPARA* Leu162Val polymorphism

Several polymorphisms have been found in the human *PPARA* gene, one of which being a C→G transversion in axon 5 at position 484 which leads to a substitution of valine for leucine at codon 162 (Leu162Val) (Tai et al, 2002). The frequency of the minor allele (Val162) is 0.042 in Europeans (http://www.ncbi.nlm.nih.gov/snp; build 132 accessed 15/12/10).

Many studies have examined the role of *PPARA* Leu162Val polymorphism in the variability of lipids and apolipoprotein concentrations. The minor allele Val162 has been associated with higher concentrations of Apo B and LDL-C (Vohl et al, 2000; Tai et al, 2002;Tanaka et al, 2007); HDL-C; Apo A1 (Flavell et al, 2000); apoC-III (Tai et al, 2002); higher serum TAG; lower HDL-C concentration, and higher subcutaneous fat volume in White males (Uthurralt et al, 2007), with lower BMI in subjects with T2D and healthy controls (Evans et al, 2001). The Val162 has also been associated with higher TC: HDL-C, and TAG: HDL ratios (Manresa et al, 2006); higher TAG and apoC-III in African Americans, but not Caucasians (Shin et al, 2008) and with lower TAG concentrations (Nielsen et al, 2003). The Val162Val homozygotes have significantly higher TC and TAG concentrations compared to Leu162 carriers (Sparsø et al, 2007). However, the Leu162Val SNP has not been associated with any of the lipid profiles (Pishva et al, 2009), BMI, body fat composition, insulin sensitivity, or insulin secretion in three European cohorts: TULIP, TUEF and the LURIC (Silbernagel et al,

2009). Furthermore, it is not linked with the lipid profiles of patients with T2D in the Go-DARTS study (Doney et al, 2005).

In a study population in which subgroups with or without T2D were compared, carriage of the Val162-allele was associated with reduced concentrations of HDL-C and a reduced risk of CVD in the DM group. Only in Val162 carriers is fibrate treatment in the DM group associated with a reduced risk of CVD and a significantly increased CVD risk in the absence of DM (Tai et al, 2006). This may suggest that the beneficial effect of the Val162-allele in subjects receiving fibrate treatment depends on ligand concentration. The following section reviews the influence of dietary ligands on *PPARA* Leu162Val.

1.8.2 *PPARA* Leu162Val polymorphism and diet

A similar concentration-dependent effect was reported for endogenous ligands by Tai et al. (2005). When PUFA intake was less than 4%, carriers of Val162-allele had higher levels of plasma TAG in comparison with Leu/Leu homozygotes. However, when PUFA intake was more than 8%, Val162-allele carriers had lower concentrations of plasma TAG than Leu/Leu homozygotes (Tai et al, 2005).

Carriers of the Val162-allele have been characterised by higher plasma Apo B and TAG concentrations (Robitaille et al, 2004). This study also reported a significant gene-diet interaction between the *PPARA* Leu162Val genotype and total fat intake in determining waist circumference. They reported that in Leu/Leu homozygotes, waist circumference increased with a higher intake of dietary fat, but no significant interaction was found in determining TC, LDL-C, HDL-C or Apo B concentrations. A study by Caron-Dorval et al. (2008), examined whether n-3 PUFA induced changes in CVD risk factors are influenced by *PPARA* Leu162Val polymorphism. This study reported that carriers of the Val162-allele exhibited an increase in the C-reactive protein (CRP), while a decrease CRP was reported in Leu126-allele carriers with n-3 PUFA supplementation. The ARIC study reported a significant genotype and n-6 PUFA intake in White subjects, with a reduction in TC and LDL-C in Val162Val subjects. Similar genotype-phenotype association was reported in African-Americans but with n-3 PUFA intake in determining LDL-C and TC (Volcik et al, 2008). In healthy men, following a high P:S diet, Val162-allele carriers had lower Apo A1 concentrations, compared to Leu/Leu

homozygotes (Paradis et al, 2005). These findings suggest that associations of the lipid profile with the *PPARA* Leu126Val can be modulated by dietary intake of fat. Reports of the relative activities of the Leu162 and Val162 PPARα isoforms *in vitro* have been contradictory, possibly owing to dependence on ligand concentration. Sapone et al. (2000) found Val162 allele had greater activity than Leu162 at high, but lower activity at low ligand concentration. Flavell et al. (2000) originally found Val162 showed greater transactivation in a reporter construct. However, recently Rudkowska et al. (2010) found transcription to be higher in Leu162- than Val162-constructs containing the *LPL* PPRE, after *n*-3 fatty acid transactivation. They also found an inverse correlation between LPL activities and plasma TAG levels in Leu162 homozygotes but not in Val162 carriers (Rudkowska et al, 2009), suggesting that Val162 has lower transactivational ability than Leu162 under physiological conditions.

1.9 Hypothesis

Understanding the nature of multiple gene-gene interaction and gene-environment interactions is pivotal in understanding the causes and progression of metabolic syndrome and its management (Roche et al, 2005). As outlined in the introductory paragraph the aim of this thesis was to examine the influence of dietary lipids and variations in *ADIPQQ* gene on serum adiponectin concentrations. As adiponectin expression could be modulated by PPARs it was decided to include *PPARA* and *PPARG* genotypes in the study.

It was hypothesised that the type and amount of fatty acids in the diet would influence adiponectin concentrations and that this may be modulated by common polymorphisms in *ADIPQO, PPARG* and *PPARA* genes.

Chapter 2

Subjects, Materials and Methods

2.1 Intervention studies

2.1.1 RISCK

2.1.1.2 Subjects

The RISCK (Reading, Imperial, Surrey, Cambridge, King's) study aimed to test the influence of specific dietary changes on cardiovascular risk factors associated with metabolic syndrome, among subjects at risk of metabolic syndrome. RISCK tests the impact of four different diets on CVD risk, with a change in insulin resistance as the primary outcome. The four treatments were diets high in MUFA or low fat, each subdivided into a high or low glycaemic index group. The principle investigators are Dr. Susan Jebb (Medical Research Council Human Nutrition Research Centre, Cambridge), Prof. Gary Frost (Imperial College London), Prof. Tom Sanders (King's College London), Dr. Julie Lovegrove (University of Reading), and Dr. Bruce Griffin (University of Surrey). Recruitment onto the trial commenced in August 2004 and was completed in November 2006.

Men and women (age range: 30-70 years) recruited from the general population, through media advertisements, attended a clinic in a fasting state at the participating centres (University of Reading, Imperial College London, University of Surrey and the Medical Research Council Human Nutrition Research Centre [MRC-HNR], University of Cambridge and King's College London). Eligibility for entry to the study was assessed by a point system and implementation of exclusion criteria described previously (Jebb et al, 2010). 47.5% of the subjects had the metabolic syndrome according to the criteria of the IDF (Alberti et al, 2006). 548 subjects completed the study and self-reported ethnicity was recorded as White, South Asian, Black African, or other.

2.1.1.3 Study design

A parallel 2 × 2 factorial design compared with a control intervention was used. Subjects were randomly assigned to treatments using a computer-based minimization procedure to balance assignment by age, sex, waist, and HDL-C as described previously (Jebb et al, 2010). Power calculations were based on 113 subjects per group completing the study to give 80% power to detect a difference in means of 1 ($\times 10^{-4}$ mL · μU^{-1} min^{-1}) in the index of Si at $P = 0.005$. The final sample size, allowing for dropout of 15%, was 130 per treatment group with equal numbers recruited at each of the five centres. The intervention diets were planned to provide similar intakes of dietary energy, but to vary in the amount and type of fats and carbohydrates. At screening, unweighed 4-d food diaries (3 weekdays and 1 weekend day) were collected to record the habitual diet. Nutrient intakes were estimated by using the food-composition database software Diet in Nutrients out (DINO) as described previously (Moore et al, 2009). All participants followed a 4-week run-in period during which they consumed a high-saturated fat 'reference diet' before being randomised to the reference diet or one of four isoenergetic dietary interventions designed to lower saturated fat.

For the purposes of the genetic investigation studies the dietary intervention groups differing in carbohydrate quality were combined to focus the analyses on the manipulation of dietary fat, from which the impact on adiponectin concentrations, insulin sensitivity and lipid profile was expected to be greater. The resulting three dietary groups were: high saturated fat 'reference diet' (HS) designed to reflect saturated fat intake in a 'Western' diet, 'high-MUFA diet' ;(HM) in which SFA was reduced and replaced with MUFA and 'low-fat diet' (LF), in which SFA was reduced through replacement of total fat with carbohydrate. The target intake for total fat was 38% of energy in the HS and HM diets and 28% of energy in the LF diets, with carbohydrate intakes of 45% and 55% of energy respectively. The HM and LF diets were designed to reduce dietary SFAs to 10% of energy with a planned MUFA intake of 20% of energy in the HM diets and 12% of energy in the LF and HS reference diet. Measurements made after the run-in diet are referred to as 'baseline'. All participants followed their randomly prescribed diets for 24 weeks, after which a further blood sample was collected and anthropometry measured. Ethical approval for the RISCK study (ISRCTN29111298) was granted from the National Research Ethics Service and

written informed consent from participants was obtained, including subsequent genetic analyses.

2.1.2 MARINA

2.1.2.1 Subjects

MARINA (**M**odulation of **A**therosclerosis **R**isk by **I**ncreasing dose of **N**-3 fatty **A**cids) study was designed to determine the effect of intakes of (n-3) LCP, equivalent to intakes provided by 1, 2 and 4 portions of oily fish per week, on endothelial function and established predictors of risk of CVD. The principal investigators are Prof. Tom Sanders, Dr. Wendy Hall, Dr. Fiona Lewis, Dr. Paul Chowienczyk and Dr. Zoe Maniou. The study commenced in May 2008 and was completed in July 2009.

Participants in the MARINA study were non-smoking men and women aged between 45-70 years, recruited through media advertisements. Potentially eligible subjects were screened to obtain measures of BP, height, weight, % body fat and waist circumference. Exclusion criteria included history of CVD, stroke, cancer, type 1 diabetes, chronic renal, liver or bowel disease, smoking, excess alcohol intake, pregnancy, weight change of > 3 kg in the preceding 2 months, BMI < 20 and > 35 kg/m^2, untreated high BP or raised cholesterol. Sample size calculations were based on flow mediated diameter (FMD) as the primary outcome. In order to detect a 20% change in mean FMD, a sample size of 72 subjects/group was required for 90% power. The significance level was set at 0.01%. Hence the final sample size, allowing for a dropout rate of 20%, was 90 per treatment group. The aim was therefore to recruit 360 subjects to allow for a non-completion rate of 20% (292 completing) (Sanders et al, 2011).

2.1.2.2 Study design

The MARINA study is a randomised double-blind parallel design to test the effects of three dose levels of DHA and EPA versus placebo primarily on endothelial function. Secondary outcomes include lipid variables associated with risk of CVD: plasma TC, LDL-C and HDL-C and TAG concentrations. Each treatment comprised an initial run-in period of 4 weeks, taking olive oil (British Pharmacopoeia specification) placebo capsules whilst following dietary advice to restrict oily fish intake. Baseline measurements of outcome variables were made at the end of the run-in period.

Participants were then randomly allocated to one of the four treatments using age, gender and ethnicity. The 12 months dietary intervention phase involved supplementation with encapsulated (n-3) PUFA, EPA and DHA, at three different doses (0.45, 0.9 and 1.8 g/d), compared with placebo. The lowest level of intake is based on the Scientific Advisory Committee on Nutrition Recommendation to consume two portions of fish per week, one of which should be an oily fish, which provides an intake of 3.2 g/week or 0.45 g/d. Further measurements of the outcome variables were made after 6 months and at the end of the 12 months intervention. Compliance to the dietary intervention was assessed from capsule counts and by measuring changes in the proportions of EPA and DHA in erythrocyte lipids. Details on the supplements used in the can be found in **Appendix 2.1**.The study received NHS R&D approval from the Guy's and St Thomas' Hospitals Foundation Trust (ISRCTN66664610).

The design and recruitment to the RISCK and MARINA studies were conducted by the study group. The author's role was to extract the DNA for both studies and preformed genotyping on the selected SNPs and the statistical analysis of data.

2.2 Materials

2.2.1 DNA extraction:

DNA was extracted from the buffy coats using the Illustra blood genomicprep spin kit, (GE Healthcare, UK).

2.2.1.1 Materials for DNA extraction

Item	Supplier
Proteinase K	GE Healthcare, UK
Lysis buffer	GE Healthcare, UK
Wash buffer	GE Healthcare, UK
Elusion buffer	GE Healthcare, UK
Illustra blood mini column	GE Healthcare, UK
Collection tubes	GE Healthcare, UK
96-well microplates	Qiagen, Crawley, Surrey, UK
Sterile plate covers	Qiagen, Crawley, Surrey, UK

2.2.1.2 Equipment

Item	Supplier
Microcentrifuge that accommodates 1.5 ml microcentrifuge tubes	DJB Labcare Ltd, Buckinghamshire, UK
Water bath or heat-block for 70^{o}C incubation	Grant Operation
Vortex mixer	Fisherbrand, Fisher Scientific, UK

2.2.2 Polymerase Chain Reaction (PCR)

2.2.2.1 Reagents and chemicals

Item	Supplier
10 x PCR Buffer without $MgCl_2$	Sigma-Aldrich, Gillingham, UK
Nucleotides dATP, dCTP, dGTP, dTTP	Invitrogen Ltd, Paisley, UK
Primers	Sigma-Aldrich, Gillingham, UK
$MgCl_2$	Sigma-Aldrich, Gillingham, UK
RedTaq Genomic DNA Polymerase without $MgCl_2$	Sigma-Aldrich, Gillingham, UK

2.2.1.2 Equipment and materials

Item	Supplier
DNA Engine TETRADTM2 Peltier thermocycler	Bio-Rad Laboratories
96-wells (8x12) microplates	ABGene, Thermo Fisher scientific
Rubber sealing mats	ABGene, Thermo Fisher scientific

2.2.3 Electrophoresis and gel imaging

2.2.3.1 Reagents

Item	Supplier
Agarose	Sigma-Aldrich, Gillingham, UK
10XTBE Buffer	Invitrogen Ltd, Paisley, UK
Ethidium bromide	Sigma-Aldrich, Gillingham, UK
Loading dye	Sigma-Aldrich, Gillingham, UK
100 bp ladder	Invitrogen Ltd, Paisley, UK

2.2.3.2 Equipment

Item	Supplier
Small horizontal electrophoresis tank	Fisherbrand, Fisher Scientific, UK
GeneGenius Imaging System	Syngene, Cambridge, UK
GeneSnap software	Syngene, Cambridge, UK
Sony-UP-D895 digital graphic printer	Sony

2.2.4 Pyrosequencing

2.2.4.1 Reagents

Item	Supplier
Ultrapure water (18mΩ),	Invitrogen Paisley, UK
70% ethanol	Invitrogen Paisley, UK
Denaturating solution	Invitrogen Paisley, UK
10 x washing buffer	Biotage, Uppsala, Sweden
Binding buffer	Biotage, Uppsala, Sweden
Annealing buffer	Biotage, Uppsala, Sweden
Sepharose-streptavidin beads	GE Healthcare, UK
Sequencing primer	Sigma-Aldrich, Gillingham, UK
Reagent kit 50x96 (enzyme, substrate and dNTPs)	Biotage, Uppsala, Sweden

2.2.4.2 Equipment

Item	Supplier
Pyrosequencer PSQ HS 96	Biotage, Uppsala, Sweden
Pyrosequencing vacuum preparation tool	Biotage, Uppsala, Sweden
Filter probes	Biotage, Uppsala, Sweden
Pyrosequencing PSQ HS 96 plate	Biotage, Uppsala, Sweden
PSQ HS CDT dispensing tip holder	Biotage, Uppsala, Sweden
PSQ HS reagent dispensing tips	Biotage, Uppsala, Sweden
Barnstead Thermolyne Hot block	Thermo Fisher Scientific, UK
Vortex Gene-2 Horizontal shaker	Scientific Industries

2.3 Methods

2.3.1 Blood analytic methods

Blood samples for analysis were drawn after a minimum 8-hour overnight fast and serum was stored at -45 °C until analysed. The author was not involved in analysing any of these variables.

2.3.1.1 Adiponectin (RISCK study)

Serum adiponectin analysis was carried out by Duncan Talbot at Unilever (Sharnbrook, UK) using AutoDELFIA-time-resolved-fluorescence based immunoassay (Perkin Elmer, Cambridge, UK). Tests of intra- and inter-assay coefficients of variance (CV) were performed in replicates of 25. Intra- and inter-assay % CV respectively were as follows: 0.95 μg/ml human adiponectin: 7.2, 8.8; 2.88 μg/ml: 7.5, 8.2; 8.60 μg/ml: 5.0, 1.4. More detailed methods can be found in **Appendix 2.2**.

2.3.1.2 Blood lipids

Analyses of TC, HDL-C and TAG were carried out by Dr Roy Sherwood at the Department of Clinical Biochemistry, King's College Hospital. TC was determined using an enzymatic method using cholesterol esterase, cholesterol oxidase and peroxidase in a chemiluminescent reaction to produce a red quinoneimine dye. The increase in absorbance was measured as an endpoint reaction at 505/694 nm. For RISCK samples, inter-assay CVs were 1.1, 1.5 and 1.0 at 3.9, 5.2 and 5.7 mmol/L respectively. For MARINA samples inter-assay CVs were 1.1, 1.5 and 1.0 % at 3.9, 5.1 and 5.7 mmol/L respectively. HDL-C was analysed using a two-step automated procedure (Bayer Advia Direct HDL-C method). For RISCK samples CVs were 2.2, 2.1 and 2.5 at 0.91, 1.39 and 1.95 mmol/L respectively and for MARINA samples CVs were 2.2, 2.1 and 2.5 % at concentrations of 0.91, 1.39 and 1.95 mmol/L respectively. TAG was measured using an enzymatic assay (The Bayer Advia method). For RISCK samples, CVs were 2.5 and 1.5 at 1.32 and 2.36 mmol/L respectively and for MARINA samples CVs were 2.5 and 1.5 % at concentrations of 1.32 and 2.36 mmol/L respectively. LDL-C was calculated using the Friedwald formula if fasting plasma TG concentrations were < 4.49 mmol/L. The formula used was: LDL-C = TC - HDL-C - (TAG / 2.2) (Friedewald et al, 1972).

For RISCK samples, plasma Apo B and Apo A1 were measured at the University of Surrey using commercially available kits (Randox, UK) that employ immuno-turbidimetric assays. The proportion of small dense LDL was measured by ultracentrifugation of the LDL fraction on an iodixanol gradient at the University of Surrey. Fasted plasma phospholipid fatty acids were measured by GC at the University of Reading as described by (Moore et al, 2009).

For MARINA samples, erythrocyte membrane phospholipid fatty acid composition measured using capillary gas liquid chromatography as described by Sanders et al. (2006). Mean inter-assay and intra-assay CVs were 3% and 2% respectively.

2.3.1.3 Insulin sensitivity (RISCK study)

Insulin sensitivity was measured by the intravenous glucose tolerance test (IVGTT) and insulin was measured at the Nutritional Biochemistry Laboratory at MRC HNR. Glucose was measured by the hexokinase-glucose-6- phosphate dehydrogenase method, using the Dimension® clinical chemistry system (Dade Behring, Milton Keynes, UK). Typical inter-assay CV was 2.4%. Insulin samples were assayed on a Roche Elecsys analyser using an electrochemiluminescence immunoassay (Roche, Indianapolis, USA). Inter-assay CVs were 4.5% at 169ρmol/L and 3.6% at 552ρmol/L. Insulin resistance was indirectly assessed by estimated HOMA2-IR using software from the Diabetes Trials Unit, University of Oxford (Internet: http://www.dtu.ox.ac.uk/homa). More detailed methods can be found in **Appendix 2.3.**

2.3.2 Anthropometry

Weight was measured after an overnight fast to the nearest 0.1 kg using digital scales. Height was measured to the nearest 0.1 cm using a wall-mounted stadiometer. Waist circumference was measured to the nearest 0.1 using a measuring tape. Body composition was assessed using a Tanita BC-418 segmental body composition analyser (Tanita Corporation of America, Inc., Illinois USA). More detailed methods can be found in **Appendix 2.4.**

2.3.3 Seated blood pressure

Blood pressure and heart rate was taken using an OMRON 705CPII or equivalent auto upper arm blood pressure monitor. More detailed methods can be found in **Appendix 2.5.**

2.3.4 DNA extraction

DNA extraction from buffy coats of 466 RISCK subjects and 310 MARINA subjects was carried out using an Illustra blood genomic prep mini spin kit (GE healthcare, UK). Buffy coats in EDTA had been frozen since preparation of blood samples. Genomic DNA was purified from 200 µl buffy coat. This process was carried out according to manufacturer's instructions. This includes the below steps:

1- **Blood cell lysis**: 20 µl of Proteinase K was added into the bottom of a 1.5 ml microcentrifuge tube. This digests protein, removing contamination and inactivating nucleases that might otherwise degrade the DNA during purification. This was followed by adding 200 µl of the buffy coat and then 400 µl lysis buffer into the same tube. The tube was mixed well for 15 seconds and incubated at room temperature for 10 minutes.
2- **Genomic DNA binding:** a mini column was assembled in a collection tube. The sample was loaded on to the centre of the column and was centrifuged. The flow through in the collection tube was discarded. The column was placed back inside the collection tube.
3- **Wash:** to ensure complete cell lysis and to denature any residual protein; 500 µl of lysis buffer was added to the column and centrifuged. And again, flowthrough in the collection tube was discarded.
4- **Wash and dry:** into the column, 500 µl of wash buffer was added. This was centrifuged for 3 minutes. The collection tube and flow through were discarded.
5- **Elution:** the purification column was transferred into a fresh DNAse- free microcentrifuge tube. Followed by adding 200 µl of 70 ^{o}C preheated elusion buffer on the centre of the column. This was centrifuged for 1 minute. Purified genomic DNA was stored at -20 ^{o}C.

2.3.4.1 Quantitation of DNA samples

The quality of the DNA samples was checked by NanoDrop (DNA quality analyses). NanoDrop accurately measures dsDNA samples up to 3700 ng/µl without dilution. It automatically detects the high concentration and utilizes the 0.2mm path length to calculate the absorbance as shown in **Table 2.1.** For NanoDrop spectrophotometry, 1.0 µl of sample was used to quantify DNA concentration. A blank of nuclease-free water, was used to optimise the instrument. Sample pedestals were first cleaned with nuclease-free water and ethanol prior to use.

Table 2.1 Sample of DNA quality analyser

Sample ID	User ID	Date	Time	ng/ul	A260	A280	260/280	260/230	Constant	Cursor Pos.	Cursor abs.	340 raw
267	Default	15/07/2009	16:12	93.51	1.870	1.051	1.78	1.16	50.00	230	1.611	0.031
268	Default	15/07/2009	16:12	22.13	0.443	0.245	1.80	0.67	50.00	230	0.656	0.010
269	Default	15/07/2009	16:13	50.57	1.011	0.522	1.94	1.32	50.00	230	0.768	-0.017
270	Default	15/07/2009	16:13	68.06	1.361	0.733	1.86	1.31	50.00	230	1.037	-0.006
271	Default	15/07/2009	16:14	71.78	1.436	0.764	1.88	1.51	50.00	230	0.953	-0.007
272	Default	15/07/2009	16:14	59.28	1.186	0.644	1.84	1.15	50.00	230	1.029	0.014

260/280: the ratio of absorbance at 260 and 280 nm is used to assess the purity of DNA. A ratio of 1.8 is accepted as pure for DNA. If the ratio is lower, it may indicate the presence of contaminants such as protein or phenol that absorb strongly at or near to 280 nm.

After checking the quality of the extracted DNA, samples were diluted to 10 ng/µl. The high quality, intact genomic DNA from the buffy coat fraction of the RISCK subjects was frozen and stored in 5 x 94 wells and 1 x 60 on 96 well DNA plates, and 3 x 94 wells and 1 x 55 wells on 96 well DNA plates for MARINA subjects. Samples were defrosted as needed for analysis.

2.3.5 Polymerase chain reaction

PCR is a technique where a specific sequence of DNA is amplified within the genome. Two oligonucleotide primers are required to bind to the target DNA segment which will allow the selective amplification of the target sequence. PCR was used to generate the template for SNP genotyping by Pyrosequencing.

There are three cycles in the PCR which are needed to be carried out at different temperature: PCR stages

1- **Denaturation** separates the DNA chains in the double helix.
2- **Annealing** was optimized for each DNA to allow the designed primers bind to the ends of the DNA strands.
3- **DNA synthesis** to allow the Taq polymerase to make a complete copy of the templates in the presence of deoxynucleotide triphosphates (dNTPs) dATP, dCTP, dGTP, dTTP.
4- The PCR product can then be stored at 4^0C.

2.3.5.1 Primers selection

Primers for the *PPARA* Leu162Val (rs1800206) were selected by entering approximately 200 base pair (bp) of 5' and 3' flanking sequence (http://www.ncbi.nlm.nih.gov/snp; build 132 accessed 15/12/10) in to the Pyrosequencing assay design software program (PSQHS96 Machine SNP Analysis, Program 1.2.1). Suitable primer options were generated with quality scores up to 100. The primer set selected for *PPARA* Leu162Val scored 97 as shown in **Table 2.2**. The primer set represented high quality and neither set overlapped with any other SNP located in the flanking sequence.

Table 2.2 Pyrosequencing assay design analysis for *PPARA* L162V

Assay Name	*PPARA* L162V	**Created by**	RMWO46586301\Sandra
Assay Type	Genotyping	**Created date**	03/04/2009 12:25
Direction	Forward	**Changed date**	03/04/2009 12:26
Description			
Notes			

Primer Set 1			**Score:97 Quality: High**		
Primer	**Id**	**Sequence**	**Bp**	**Tm, °C**	**%GC**
PCR	F1	GCCAGTATTGTCGATTTCACAAGT	24	70.4	41.7
PCR	R1	TTACCTACCGTTGTGTGACATCC	23	69.9	47.8
Sequencing	S1	TCGATTTCACAAGTGC	16	50.7	43.8

Target Polymorphisms	Position1
Sequence to Analyse	[C/G]TTTCT GTCGGGATGT CACACAACGG

ADIPOQ SNPs primers were those previously selected for a previous study in this laboratory (Kyriakou et al 2008). The relative positions of tSNPs with respect to the first coding base in exon 2 are shown in parentheses: rs17300539 (-11391 G/A), rs182052 (-10066 G/A), rs16861209 (-7734 C/A), rs1501299 (+276 G/T).

Table 2.3 *ADIPOQ* SNPs primers

dbSNP	Alleles	Template PCR primers
rs17300539	-11391 *G/A*	F: 5'-biotin-CTGGGGTCGTAATTTAATTCATCA-3' R: 5'-TTCTTGGCACGCTCATGTT S: 5'-GGGCAGGATCTGAGC-3'
rs182052	-10066 *G/A*	F: 5'-biotin- GTAGTGTGGGAGTGGATACAGGT-3' R: 5'-AATCGAATTGGACTTCATCTGTG-3' S:5'-CTGCTACAGAGCGAACT-3'
rs16861209	-7734 *C/A*	F: 5'-biotin-TGTGTTGGGCATGGAGATATT-3' R: 5'-GAGGTTGCAGTGAGCTGAGAT-3' S: 5'-TGGCAGTTTCACACG-3'
rs1501299	+276 *G/T*	F: 5'-biotin-GGCCTCTTTCATCACAGACCT-3' R: 5'-GCTTTGCTTTCTCCCTGTGTCT-3' S: 5'-GGCCTTAGTTAATAATGAAT-3'

2.3.5.2 PCR optimization:

Determination of the optimal temperature is crucial, as high temperatures prohibit the primers from annealing to the DNA strands. Optimizing the magnesium chloride ($MgCl_2$) concentration is necessary to ensure primer annealing specificity. A deficient of $MgCl_2$ can result in an inadequate yield of PCR product, however poor primer specificity may result from an excess of $MgCl_2$.

Using the selected primers, and pooled control DNA, the optimum annealing temperature and $MgCl_2$ concentration were determined for each of the SNPs. This was done by setting up $MgCl_2$ titration on a block with a temperature gradient facility for the annealing cycle. The *ADIPOQ* SNP PCR was optimized previously by Ms Laura Collins (Kyriakou et al, 2008). *PPARA* Leu162Val PCR was optimised as follows:

In a PCR plate, 1 µl of pooled DNA (10ng/µl) was added into columns 1, 4, 5, 6, 7 and 8 of the first three rows. Each column was labelled with one of the annealing temperatures, and each of the rows with one of the three $MgCl_2$ concentrations as shown in **Figure 2.1.**

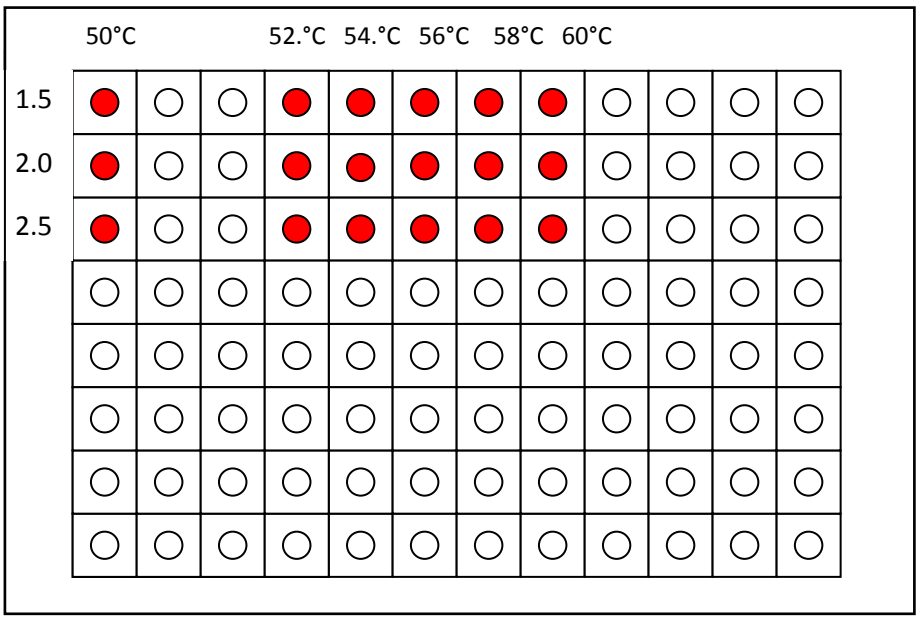

Figure 2.1 Representing the labelling of the PCR plate *PPARA* Leu162Val

For optimization, the PCR mixture was made up in a single 500µl Eppendorf tube without $MgCl_2$ to yield a total of 270µl (**Table 2.4**). The PCR volume was split equally into three separate 500µl Eppendorf tubes (90µl per tube).

Table 2.4 PCR reagents and their volumes without $MgCl_2$

PCR reagent	Volumes (µL)
Sterile water	229.2
10x PCR buffer	30
dNTP mix ACGT	2.4
Forward primer	1.2
Reverse primer	1.2
RedTaq polymerase	6.0
Total	270

Into each of the three tubes a 10µl $MgCl_2$ solution of varying concentrations was inserted; this was achieved by adding different proportions of sterile water and 25mM $MgCl_2$ to produce the relevant $MgCl_2$concentration **Table 2.5**. This addition of $MgCl_2$ raised the total volume of PCR mixture to the final volume of 100µl.

Table 2.5 Volume of reagents used for the various $MgCl_2$ concentrations

Concentration of $MgCl_2$ (mM)	25 mM $MgCl_2$ (µL)	Sterile water (µL)
1.5	6	4
2	8	2
2.5	10	0

The PCR mixture was thoroughly mixed and then aliquoted into the required wells in the PCR plate, 10µl was added per well (preloaded with DNA). A rubber mat was used to seal the PCR plate and it was placed into the Tetrad thermocycler. Once completed the plate was stored at 4˚C while preparations were made for gel electrophoresis

2.3.6 Electrophoresis and Gel Imaging:

Following PCR optimisation, gel electrophoresis was used to determine the length of the DNA fragments. Small 1% agarose gels (135x156mm) were made up to run the sample of each optimization array. The gel was prepared by adding 0.7g agarose powder to 100ml 1 X Tris/Borate/EDTA (TBE) buffer; the mixture was then heated in the microwave for about 10 minutes until the agarose powder was fully dissolved. After the gel was cooled down, 10 µl of ethidium bromide was added. The gel was then poured into the gel tray (sealed with autoclaving tape). Two combs were inserted into the apparatus in order to create two rows of 16 wells. The gel was left to set for about 30 minutes at room temperature. Once solidified, the combs were gently removed and the gel was placed into an electrophoresis tank. This was followed by pouring 1x TBE buffer into the tank, facilitating immersion of the gel.

Into the first well of each row, 5µl of DNA ladder was inserted. 2µl of loading dye was added to the 10µl of PCR product present in each well of the PCR plate. They were then extracted from the PCR plate and inserted into the respective well of the gel. Loading buffer helps to stain the DNA fragments and enable the PCR product/loading dye mix to sink into the wells.

On completion the gel was carefully removed from the tank and placed in the gel documentation apparatus for visualization and recording of the image. The image was photographed and bands of DNA fragments examined to determine at which temperature and $MgCl_2$ concentration the brightest band was produced i.e. the PCR conditions at which the greatest number of DNA fragments were amplified.

After the electrophoresis, the gel was taken to the gel documentation apparatus for visualization under an ultraviolet (UV) illuminator. The image was photographed and printed out. Bands of DNA fragments were examined to determine at which temperature and $MgCl_2$ concentration the brightest band

2.3.7 PCR

After optimizing the annealing temperature and the $MgCl_2$ concentration, micro plates were prepared for the template PCRs for each SNP. A total volume of 1000 µL of PCR mix was prepared for 96 well micro plates for each SNP as following:

Table 2.6 PCR mixture for 96 well microplate

SNP	Sterile water (µl)	PCR buffer (µl)	ACGT mix (µl)	F primer (µl)	R primer (µl)	$MgCl_2$ (µl)	Red taq polymerase (µl)
ADIPOQ - 10066 G/A	804.0	100	8.0	4.0	4.0	60.0	20.0
ADIPOQ - 7734 C/A	784.0	100	8.0	4.0	4.0	80.0	20.0
ADIPOQ - 11391 G/A	784.0	100	8.0	4.0	4.0	80.0	20.0
ADIPOQ + 276 G/T	804.0	100	8.0	4.0	4.0	60.0	20.0
PPARA Leu162Val	804.0	100	8.0	4.0	4.0	60.0	20.0

Into each well of the PCR plate, 10 µl of PCR mix was pipetted. This was followed by pipating 1.5 µl of the DNA sample into the appropriate well. The plate was then covered and placed into Tetrad Thermocycler and programmed for the specific conditions required for the SNP.

Table 2.7 PCR conditions for the selected SNPs

SNP	PCR cycling conditions
ADIPOQ -10066 G/A and -7734 C/A	1 cycle at 94 ℃ for 6 minutes; 50 cycles at 94℃ for 1 minute; 61 ℃ for 30 seconds; 72℃ for 30 seconds; 1 cycle at 72℃ for 10 minutes.
ADIPOQ -11391 G/A and + 276 G/T	1 cycle at 94 ℃ for 6 minutes; 50 cycles at 94℃ for 1 minute; 59 ℃ for 30 seconds; 72℃ for 30 seconds; 1 cycle at 72℃ for 10 minutes.
PPARA Leu162Val	1 cycle at 94 ℃ for 6 minutes; 50 cycles at 94℃ for 1 minute; 54.0 ℃ for 45 seconds; 72℃ for 1 minutes ; 1 cycle at 72℃ for 10 minutes.

The completed PCR was the removed and stored in a 4^0C fridge ready for the Pyrosequencer.

2.3.8 SNP genotyping by Pyrosequencing:

Pyrosequencing is a real time sequencing strategy based on the release of pyrophosphate (PPi) during enzymatic DNA synthesis (Ronaghi, 2001). The general principal of the Pyrosequencer is that a polymerase catalyses incorporation of nucleotide(s) into a nucleic acid chain. This incorporation will cause the release of PPi molecule (s) and subsequently the conversion of ATP by ATP sulfurylase. As a result of this, light is produced in the luciferase reaction, during which a luciferin molecule is oxidized.

A pyrosequencing instrument was used for the selected SNPs. This is an automated, highly sensitive and quantitative genetic analysis system that is based on pyrosequencing technology. The Pyrosequencer high sensitivity light detection system enables a small DNA sample (10 µl of PCR product) to be analysed.

2.3.7.1 Principle of Pyrosequencing

A sequencing primer is hybridised to a single strand, PCR amplified, DNA template, and incubated with the enzymes (DNA polymerase, ATP sulfurylase, luciferase and apyrase) and the substrates, adenosine 5'phosphosulfate (APS) and luciferin (Ronaghi, 2001).

After the addition of the first four deoxynucleotide triphosphates (dNTP), DNA polymerase catalyses the incorporation of the dNTP into the DNA stand if it is complementary to the base in the template strand. Each incorporation events is accompanied by the release of (PPi) in an equal quantity to the amount of incorporated nucleotide (Ronaghi, 2001). PPi is then converted to ATP via ATP sulfurylase, in the presence of APS. ATP will then drive the conversion of luciferin to oxyluceferin via luciferase that generates visible light in amounts that are proportional to the amount of ATP. This light is detected by a charge coupled device (CCD) camera and seen as a peak in a program. The height of each peak is proportional to the number of nucleotides incorporated. Apyrase will continuously degrade ATP and incorporated dNTPs. This switches off the light and regenerates the reaction solution (Ronaghi, 2001).

The addition of dNTPs is performed one at a time. As the process continues, the complementary DNA strand is built up and the nucleotide sequence is determined from the signals.

2.3.7.2 Cleaning up the PCR products and preparation for sequencing:

After preparing the reagents and the equipment, a streptavidin- sepharose bead/binding buffer mix is prepared. For 100 samples, the mix was made with 220 µl sepharose beads, 4000 µl binding buffer and 3300 water. 70 µl of the bead/binding buffer was added and mixed into each the PCR products in the PCR plate; plates were shaken for 5 minutes at room temperature.

This was followed by preparing the primer/annealing buffer mix. For 100 samples, the mix was made by mixing 1540 µl of the annealing buffer with 6.6 µl of the sequencing primer. This buffer mix was then pipetted into 96 wells of pyrosequencing plate.

For strand separation, beads containing immobilised PCR templates were captured on filtered probes by lowering a vacuum prep tool in the PCR plate. Samples were then washed in 70% ethanol, denaturizing solution and washing buffer before being transferred to a Pyrosequencer plate pre-filled with the primer/annealing buffer (12 µl per well). The primers were then annealed at 80^{o}C for two minutes before running the samples in the Pyrosequencer. The volumes of the substrate, enzyme and dNTPs of the dispensing cartridge had already been calculated by the Pyrosequencer software were prepared and placed into the Pyrosequencer. The volumes varied for each of the SNP assays as shown in the table below.

Table 2.8 Volumes of enzyme, substrate and dNTPs for pyrosequencing:

SNP assay	E (µl)	S(µl)	A(µl)	C(µl)	G(µl)	T(µl)
ADIPOQ-10066 G/A	182	182	47	42	42	42
ADIPOQ -7734 C/A	182	182	42	47	42	42
ADIPOQ -11391 G/A	182	182	36	47	42	47
ADIPOQ +276 C/A	182	182	42	42	42	47
PPARA L162V	182	182	42	42	42	47

The PSQ HS96 was tested before each run to ensure functionality of all dispensing tips. At least four DNA samples from each plate were repeated to confirm consistency and accuracy of genotyping.

After completion of the pyrosequencing run, the genotype results for each pyrosequencing well were analysed; a blue well indicated a pass; red wells a fail and the yellow wells had to be manually checked to decide upon whether they were a pass or fail. The analysed results were saved on the Pyrosequencer computer. The genotypes were then transferred to an Excel spreadsheet.

2.3.9 *PPARG* Pro12Ala SNP genotyping

Unsuccessful attempts were made to genotype *PPARG* Pro12Ala by pyrosequencing. When the sequence was run through the Pyrosequencing design software, the top two primer sets scored 85 %, which is medium quality as shown in Table 2.9. The melting temperature (Tm) of the sequencing primer is 51 ºC, which requires a low (<50 ºC) annealing temperature for the sequencing primer to anneal to the DNA strand. At low temperatures, a primer is likely to anneal non-specifically. Optimisation of the template PCR using different anneal temperatures and Mg concentrations was unsuccessful, so the SNP was genotyped commercially by an alternative method.

Table 2. 9: Pyrosequencing assay design analysis for *PPARG* Pro12Ala

Primer Set 1			**Score: 85 Quality: Medium**		
Primer	**Id**	**Sequence**	**Bp**	**Tm, ºC**	**%GC**
PCR	F1	ATTACAGCAAACCCCTATTCCATG	24	71.1	41.7
PCR	R1	GTATCAGTGAAGGAATCGCTTTCT	24	68.9	41.7
Sequencing	S1	AAGGAATCGCTTTCTG	16	51.0	43.8

This SNP was genotyped in RISCK and MARINA subjects by KBiosciences (Hoddesdon, Herts) using the Kaspar system.

Chapter 3

The Influence of SNPs at the *ADIPOQ* Gene Locus and Dietary Intake of Fat on Serum Adiponectin Concentrations

3.1 Introduction

The metabolic syndrome is a complex disorder characterized by abdominal obesity, insulin resistance, hypertension, dyslipidaemia and inflammation (Tan et al, 2004). Observational evidence and intervention studies indicate that saturated fat worsens insulin sensitivity (Vessby et al, 2001), whereas the effects of monounsaturated and polyunsaturated fats on insulin sensitivity are inconsistent (Vessby et al, 2001; Djousse et al, 2011). However, individuals show varying responses to dietary intervention, suggesting that significant gene x dietary interaction occurs (Roche, 2005).

Associations with adiponectin concentration and/or the metabolic syndrome have been reported for genetic variants at the *ADIPOQ* gene locus in many studies (Vasseur et al, 2002; Jang et al, 2005). However, some of these associations could not be confirmed by others (Filippi et al, 2004). These inconsistencies may relate to interaction between the gene and environmental influences, such as dietary intake and age. Such interaction may also underpin varied individual responses to diet in general.

Adiponectin concentrations generally increase with age (Isobe et al, 2005). Results from observational or weight loss studies have suggested that diets low in carbohydrate (Pischon et al, 2005) and high in unsaturated fat (Esposito et al, 2003) might increase adiponectin concentrations. Unsaturated fatty acids are ligands for the transcription factor PPARγ (Maeda et al, 2001), which upregulates *ADIPOQ* gene expression (Iwaki et al, 2003) and directly increases serum adiponectin concentration (Maeda et al, 2001).

3.1.1 Selected SNPs

The four selected SNPs are located in the regulatory and intronic regions of the *ADIPOQ* gene as shown in **Figure 3.1**. They are all tagging SNPs, i.e. representative of a cluster of other SNPs whose alleles are on a single chromosome that are inherited together. These SNPs showed the strongest replicated associations with serum adiponectin in two cohorts previously investigated in this laboratory, using a set of 8 tagSNPs that predicted remaining SNPs in the *ADIPOQ* gene with a minimum r^2 of 0.85 (Kyriakou et al 2008).

SNP -10066 G/A (rs182052) and -7734 C/A (rs16861209) are both located in intron 1. According to NCBI dbSNP data (Internet: http://www.ncbi.nlm.nih.gov/snp build 132

accessed 15/02/11) the frequency of the common allele (G) and the rare allele (A) of SNP -10066 is 0.647 and 0.353 respectively, and for -7734 is 0.913 for the common allele (C) and 0.087 for the rare allele (A). SNP-11391 G/A (rs17300539) is located in the promoter region, the frequencies in the European population is 0.917 for the common allele (G) and 0.083 for the rare allele (A). The commonly studied SNP + 276 G/T (rs 1501299) which is found in intron 2 had a frequency of 0.733 for (G) the common allele and 0.267 of the rare allele (T).

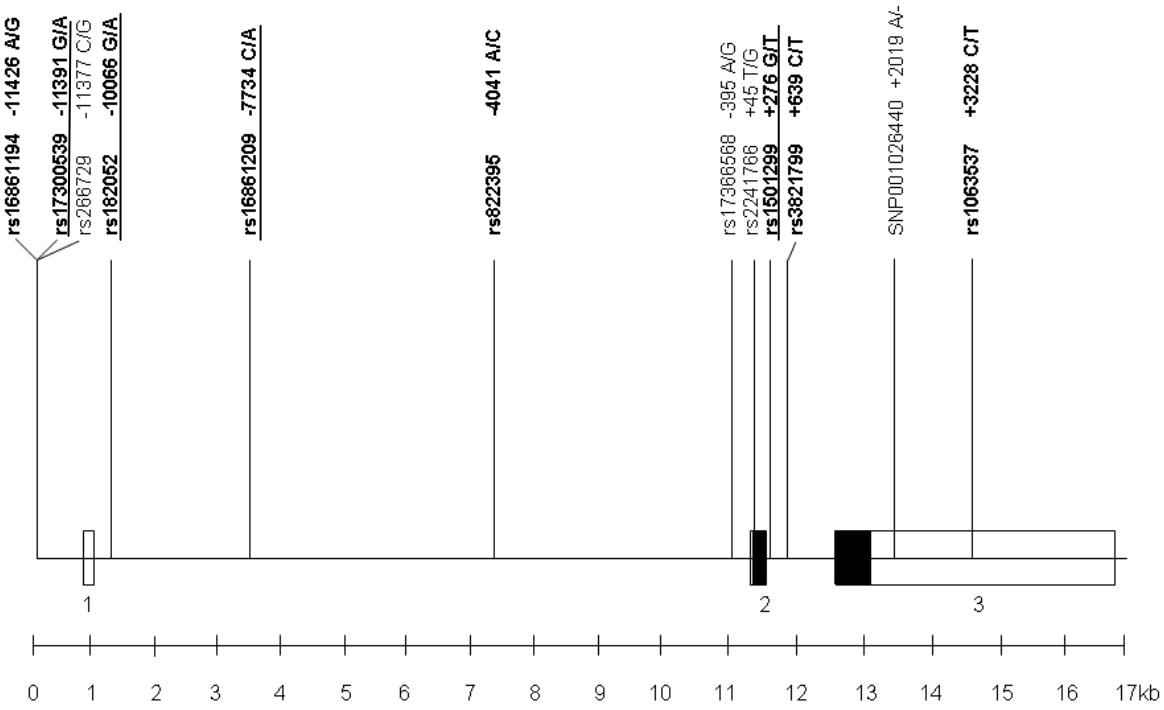

Figure 3.1 Location of the 4 selected SNPs in the *ADIPOQ* gene (underlined). Their relative positions with respect to the first coding base in exon 2 are shown. From (Kyriakou et al, 2008)

3.1.2 Hypothesis

We hypothesised that variants in *ADIPOQ* could interact with dietary intake of unsaturated fat and age to influence serum adiponectin in the absence of significant change in fat mass.

3.1.3 Aims

There is still a very limited number of studies looking at the diet-gene interaction with *ADIPOQ* variants. In view of the active role of adiponectin in the regulation of energy homeostasis, glucose and lipid metabolism, and the influence of dietary fat intake on its involvement in insulin resistance, we aimed to investigate:

1- The association between adiponectin concentrations and age, gender, BMI and ethnicity.
2- The correlation between serum adiponectin concentrations and risk factors for the metabolic syndrome.
3- Whether modulation of fat in a diet while maintaining stable weight would increase serum adiponectin concentrations
4- The association of 4 SNPs in the *ADIPOQ* gene (-10066 G/A, -7734 C/A and +276 G/T) and promoter (-11391 G/A) with circulating adiponectin and metabolic syndrome risk phenotypes at baseline in 366 White European subjects.
5- The influence of dietary intake of fats on the associations of these variants with serum adiponectin and insulin sensitivity.
6- The interaction of SNP genotypes with age and gender in the determination of serum adiponectin concentration and insulin sensitivity measures after 24 weeks of dietary intervention.

3.2 Subjects and Methods

3.2.1 Subjects

This work is based on the participants of the RISCK study. Refer to **Section 2.1.1** for details of RISCK study participants.

3.2.2 Study design

Refer to **Section 2.1.1** for description of RISCK study design.

3.2.3 Blood analytic methods and anthropometry measurements:

For serum adiponectin, plasma lipids, insulin sensitivity and anthropometric measurements; refer to **Sections 2.3.1.1, 2.3.1.2, 2.3.1.3 and 2.3.2** respectively.

3.2.4 DNA extraction and SNP genotyping

Details on methods for DNA extraction from buffy coats are given in **Section 2.3.4**. Four SNPs at the *ADIPOQ* gene locus were genotyped [-11391 G/A (rs17300539), -10066 G/A (rs182052), -7734 C/A (rs16861209) and +276 G/T (rs1501299)]. Their relative positions with respect to the first coding base in exon 2 are indicated. Genotyping by Pyrosequencing (Qiagen, Crawley, Surrey, UK) was performed in the 448 participants for whom DNA was available and who had consented to genetic analysis. Primers and PCR conditions for genotyping can be found in **Section 2.3.7.** We used internal controls and accuracy, as assessed by inclusion of duplicates in the arrays, was 98%. Genotyping success rates were 98.1, 83.5, 84.9 and 92.1%.respectively.

3.2.5 Statistical analyses

All genotype distributions were tested for deviation from the Hardy-Weinberg equilibrium by a χ^2 test with 1 df ($P > 0.05$). Statistical analyses were carried out using the SPSS version 17.0 for Windows (SPSS Inc., Chicago, IL, USA). Insulin, Si, HOMA2-IR, adiponectin, TAG and HDL-C variables were log transformed to obtain better approximations of the normal distribution prior to analysis. Data were analysed by using analysis of covariance (ANCOVA); regressing on % change or follow-up measures against baseline measures with age, change in BMI and gender as covariates

and genotype and diet as fixed factors. Outliers were excluded from the ANCOVA and were defined as points > 2.5 times the interquartile range from the median on the transformed scale at baseline, follow-up, or change from baseline. All data presented in text and tables are expressed as means or geometric means (GM) ± standard deviation (SD) or 95% confidence interval (CI). Correlations are presented as Spearman's *r*. Statistical significance was taken at $P < 0.05$. Figures were drawn using Prism version 5 (GraphPad software, Inc., California, and USA).

3.3 Results

3.3.1 Baseline characteristics of subjects

A total of 548 subjects completed the RISCK study; the characteristics of the RISCK complete cohort at baseline are presented in the **Table 3.1**. The ethnic mix of subjects was typical of England and predominantly White, with about one-fifth of subjects from ethnic minorities. Based on self-reported ethnicity, individuals of White (80%), South and South East (S, SE) Asian (9.5%); Black African (8%) and 'other' (2.5%) ancestry were distinguished. After 4-week run-in on the HS diet, there were significant differences between males and females in fasting glucose, waist circumference, TAG, systolic and diastolic BP (higher in males), HDL-C, adiponectin, insulin sensitivity and body fat percentage (lower in males). The 448 individuals for whom DNA samples were available are the subjects of this study, from which participants in the 'other' ethnic subgroup were excluded. Their characteristics at baseline with respect to ethnicity, age, obesity measures, insulin homeostatic variables, adiponectin, lipid profile and blood pressure are presented in **Appendix 3.1**.

Table 3.1 Characteristics of RISCK study subjects at baseline

Phenotype	All (n = 548)	Women (n = 318)	Men (n = 230)	P
Ethnicity[1]				
South Asia (n)	52(9.5)	31(9.8)	21(9.1)	
Black African (n)	40(7.3)	28(8.8)	12(5.2)	
White European (n)	437(79.7)	247(77.7)	190(82.6)	
Others	19(3.5)	12(3.7)	7(3.1)	
Age (years)	52.4 ± 9.8	51.8 ± 9.4	53.2 ± 10.3	0.13
BMI (kg/m^2)	28.7 ± 4.8	28.8 ± 5.3	28.5 ± 3.8	0.42
Waist circumference (cm)	97.6 ± 12.3	94.3 ± 12.4	102.3 ± 10.4	<0.001
Body fat %	33.8 ± 8.5	38.9 ± 6.4	26.6 ± 5.1	<0.001
Fasting insulin (pmol/L)[2]	60.6 ± 49.5	58.7 ± 55.1	63.3 ± 40.5	0.11
Fasting glucose (mmol/L)	5.7 ± 0.8	5.5 ± 0.7	5.8 ± 0.8	<0.001
Insulin sensitivity IVGTT)((mU/L)-1 min-1)[2]	2.7 ± 2.6	2.9 ± 3.0	2.5 ± 1.9	0.001
HOMA2-IR[2]	1.3 ± 0.7	1.3 ± 0.7	1.3 ± 0.8	0.44
Adiponectin (μg/mL) [2]	9.4 ± 5.7	11.1 ± 6.2	7.5 ± 4.1	<0.001
TAG (mmol/L) [2]	1.5 ± 0.7	1.4 ± 0.6	1.6 ± 0.8	<0.001
TC (mmol/L)	5.6 ± 1.0	5.6 ± 1.0	5.6 ± 1.0	0.88
HDL-C (mmol/L) [2]	1.4 ± 0.3	1.5 ± 0.3	1.3 ± 0.3	<0.001
Apo A1 (g/L)	1.2 ± 0.3	1.3 ± 0.3	1.2 ± 0.2	<0.001
LDL-C (mmol/L)	3.6 ± 0.8	3.5 ± 0.8	3.6 ± 0.8	0.11
Apo B (g/L)	0.99 ± 0.29	0.97 ± 0.28	1.00 ± 0.31	0.27
Systolic BP (mm Hg)	129 ± 15.7	126 ± 15.1	134 ± 15.0	<0.001
Diastolic BP (mm Hg)	80 ± 9.4	77 ± 9.0	83 ± 9.0	<0.001

Data is presented for all subjects who completed the study (n = 548). Mean ± SD, or [2]GM ± SD. All variables were measured at baseline after 4-week runs-in on reference HS diet. [1]Self-reported ethnicity. Significance of differences between women and men was determined by T-test.

3.3.2 Serum adiponectin with respect to age, BMI and ethnicity

Regression analysis showed that adiponectin positively correlated with age (beta = 0.217, P <0.001) and negatively correlated with BMI (beta = - 0.161, P < 0.001). **Table 3.2** shows that there was a significant increase in geometric mean serum adiponectin concentration with age, after adjustment for BMI, gender and ethnicity. Serum adiponectin decreased with increasing BMI, after adjustment for age, gender and ethnicity. There were highly significant differences with respect to ethnicity after adjustment for BMI, age and gender. Adiponectin was significantly higher in White Europeans than in S. Asians (P = 0.001) and Black Africans (P = 0.001). Serum adiponectin concentrations were significantly higher in females (11.1± 6.2 μg/mL) than in males (8.5 ± 4.1 μg/mL) (P < 0.001). However, there were no significant interactions

between gender x age (P = 0.69), gender x BMI (P = 0.14) or gender x ethnicity (P = 0.15) in the determination of serum adiponectin concentration.

Table 3.2 Serum adiponectin concentrations at baseline stratified by age, BMI and ethnicity

Age			BMI			Ethnicity[1]		
Age group (y)	*n*	GM adiponectin (95% CI)	BMI group (kg/m^2)	*n*	GM adiponectin (95% CI)	Ethnic group	*n*	GM adiponectin (95% CI)
<40	63	8.5 (7.5,9.7)	<25	88	11.4 (10.2,12.6)	South Asian	44	7.5 (6.5,8.7)
41-50	129	8.4 (7.7,9.2)	25-29	210	9.6 (8.9,10.2)	Black	38	6.8 (5.8,8.0)
51-60	148	10.1 (9.3,11.0)	30-35	107	8.7 (7.9,9.5)	White	366	10.2 (9.7,10.8)
61-70	108	11.2 (10.2,12.4)	>35	43	8.9 (7.6,10.3)			
P-value		<0.001	*P*-value		0.002	*P*-value		<0.001
P-value adjusted[2]		<0.001	*P*-value adjusted[3]		<0.001	*P*-value adjusted[4]		<0.001

Data is presented for subjects for whom DNA samples and serum adiponectin measurements were available (*n* = 448). GM (μg/mL); 95% CI. All variables were measured at baseline after 4-week run-in on reference HS diet. [1]Self reported ethnicity. [2]*P*-values adjusted for BMI, gender and ethnicity; [3]*P*-values adjusted for age, gender and ethnicity; [4]*P*-values adjusted for age, BMI and gender. Significance of differences between age, BMI and ethnic groups was determined by ANOVA (*P* values without adjustments) or ANCOVA (*P*-values with adjustments).

3.3.3 Correlation of serum adiponectin with insulin sensitivity measures, measures of obesity and lipid profile

To establish any relationship between serum adiponectin concentrations and insulin resistance, BMI, body fat %, and lipid profile in RISCK subjects, we tested the correlation between geometric mean adiponectin and insulin homeostatic, obesity measures and lipid profile variables in all subjects at baseline. Serum adiponectin was negatively correlated with HOMA2-IR (r = -0.38; $P < 0.001$) and positively correlated with Si, measured by IVGTT (r = 0.37; $P < 0.001$). Serum adiponectin was negatively correlated with BMI (r = -0.17; $P < 0.001$) and positively correlated with body fat % (r = 0.16; $P < 0.001$). Serum adiponectin was also negatively correlated with TAG (r = -0.26; $P < 0.001$) and positively correlated with HDL-C (r = 0.52; $P < 0.001$), Apo A1 (r = 0.32; $P < 0.001$), NEFA (r = 0.25; $P < 0.001$) and TC (r = 0.11; P =0.01). No significant correlation was found between serum adiponectin and LDL-C or Apo B.

We also found the partial correlation coefficient, adjusting for BMI, age, gender and ethnicity. The correlation was decreased, but remained significant: partial r for HOMA2-IR = -0.279 ($P < 0.001$); partial r for Si = 0.323 ($P < 0.001$); partial r for TAG = -0.23 ($P < 0.001$); partial r for HDL-C = 0.46 ($P < 0.001$) and partial r for Apo A1 = 0.32 ($P < 0.001$). After adjusting for age, gender and ethnicity, partial r for BMI = -0.214 ($P < 0.001$) and partial r for body fat % = 0.187 (P = 0.001). After adjusting for age, gender, BMI and ethnicity NEFA was not significantly correlated with adiponectin concentrations.

3.3.4 Genotyping by Pyrosequencing

Pyrograms for the *ADIPOQ* -10066 G/A genotypes are shown below (**Figure 3.2).** (For explanation of methodology see **Section 2.3.8**. More results can be found **Appendix 3.2**

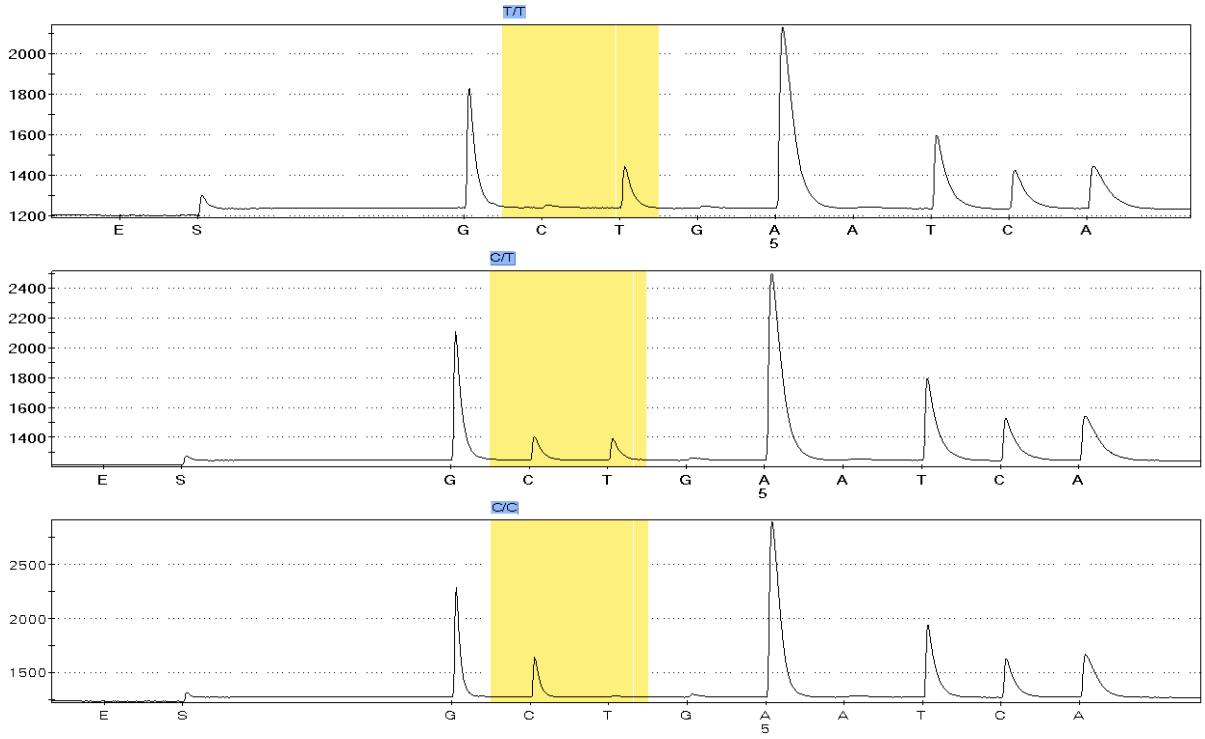

Figure 3.2 Pyrograms for -10066 G/A genotypes. Yellow blocks indicate the peaks for the analysed SNP.

3.3.5 SNP minor allele and genotype frequencies

Table 3.3 shows the minor allele frequency (MAF) and genotype frequencies of the four SNPs typed at the *ADIPOQ* locus in 466 subjects stratified by ethnic group. Genotype distributions for each SNP did not deviate from Hardy-Weinberg expectations. Allele frequencies were compared with those listed on the NCBI SNP database (Internet: http://www.ncbi.nlm.nih.gov/snp accessed 15/02/11). MAF in White Europeans were as follows: -11391 *G/A:* 0.09; -10066 G/A 0.38; -7734 C/A 0.02; +276 G/T 0.24. -11391 G/A was not expected to be present in Blacks, -10066 G/A was not as frequent as expected in Blacks (0.40 HapMap- Yoruba in Ibadan, Nigeria (YRI)) and -7734 C/A frequency was lower than expected in Europeans and Blacks (respectively 0.09 HapMap- Utah subjects of European descent (CEU) and 0.13 HapMap-YRI). +276 G/T MAFs were as expected. There were no comparable data available for S. Asians.

Table 3.3 *ADIPOQ* SNP allele and genotype frequencies

SNP		Ethnic group[1]				
		South Asian (*n*=44; 9%)	Black African (*n*=38; 8%)	White (*n*=367; 79%)	Others (*n*=17; 4%)	All (*n*=466)
-11391G/A	MAF	0.06	0.02	0.09	0.00	0.08
	11	(*n*=32; 14%)	(*n*=29; 76%)	(*n*=266; 72%)	(*n*=12; 7%)	(*n*=339; 73%)
	12	(*n*=4; 9%)	(*n*=1; 9%)	(*n*=58; 16%)	(*n*=0; 0%)	(*n*=63; 13%)
	22	(*n*=0; 0%)	(*n*=0; 0%)	(*n*=0; 0%)	(*n*=0; 0%)	(*n*=0; 0%)
-10066G/A	MAF	0.37	0.32	0.38	0.38	0.38
	11	(*n*=17; 39%)	(*n*=17; 45%)	(*n*=132; 36%)	(*n*=8; 47%)	(*n*=174; 37%)
	12	(*n*=19; 20%)	(*n*=15; 39%)	(*n*=181; 49%)	(*n*=5; 29%)	(*n*=220; 47%)
	22	(*n*=6; 14%)	(*n*=4; 10%)	(*n*=47; 13%)	(*n*=4; 23%)	(*n*=61; 13%)
-7734 C/A	MAF	0.00	0.05	0.02	0.00	0.02
	11	(*n*=38; 86%)	(*n*=30; 79%)	(*n*=280; 76%)	(*n*=14; 82%)	(*n*=362; 78%)
	12	(*n*=0; 0%)	(*n*=3; 9%)	(*n*=12; 3%)	(*n*=0; 0%)	(*n*=15; 3%)
	22	(*n*=0; 0%)	(*n*=0; 0%)	(*n*=0; 0%)	(*n*=0; 0%)	(*n*=0; 0%)
+276 G/T	MAF	0.29	0.35	0.24	0.19	0.25
	11	(*n*=20; 45%)	(*n*=14; 37%)	(*n*=196; 53%)	(*n*=11; 65%)	(*n*=241; 52%)
	12	(*n*=15; 34%)	(*n*=20; 53%)	(*n*=126; 32%)	(*n*=4; 23%)	(*n*=165; 35%)
	22	(*n*=4; 9%)	(*n*=3; 8%)	(*n*=18; 5%)	(*n*=1; 6%)	(*n*=26; 6%)

Data for all subjects with available DNA samples are shown (*n* = 466). *n* (%) refers to number of each ethnic group genotyped, as % of total. For each SNP, *n* (%) refers to number of each genotype obtained, with % frequency. [1]Self-reported ethnicity. 11 refers to subjects homozygous for the common allele; 12 refers to heterozygote subjects, and 22 refer to subjects homozygous for the minor allele.

3.3.6 *ADIPOQ* SNP genotype associations with serum adiponectin, obesity measures, insulin homeostatic variables and lipid profile at baseline

In view of the small sample size of the S. Asian and Black subgroups, we chose to focus our genetic investigation on the majority White subjects (*n* = 366). One subject did not have values for baseline or follow-up adiponectin concentrations, so we decided to exclude him. **Tables 3.4 and 3.5** show measurements of serum adiponectin, BMI, body fat %, fasting insulin, fasting glucose insulin sensitivity measured by IVGTT, HOMA2-IR, NEFA, TC, TAG, HDL-C, Apo A1, LDL and Apo B at baseline (after the 4-week run-in on HS diet), with respect to -11391 G/A, -10066 G/A, -7734 C/A and +276 G/T genotype groups. ANCOVA was used to test the association between genotypes and phenotypic values adjusted for BMI, age and gender, based on a dominant model. +276 T allele carriers had significantly higher mean serum adiponectin concentration at baseline than non-carriers (P = 0.006) and -10066 A-allele carriers had significantly

lower concentration than non-carriers (P = 0.03) before correction for multiple testing. After correction using 4-way ANOVA, the association of serum adiponectin with -10066 G/A genotype remained significant (P = 0.03), but not with +276 G/T genotype (P > 0.05). No other phenotypes were significantly associated with -10066 G/A or +276 G/T SNP genotypes at baseline (P > 0.05). No phenotypes were significantly associated with -11391 G/A or -7734 C/A SNP genotypes at baseline (P > 0.05). Neither +276 G/T nor -10066 G/A genotypes significantly interacted with age in determination of serum adiponectin concentration at baseline (P > 0.05).

Table 3.4 Serum adiponectin concentration, obesity measures, insulin homeostatic variables and lipid profile by *ADIPOQ* -11391 G/A and -10666 G/A genotypes in White Europeans

	-11391 G/A			-10066 G/A		
Phenotype	GG	GA+AA	*P*	GG	GA+AA	*P*
Male/female	125/141	17/ 41		57/ 75	95/133	
Adiponectin (μg/mL)[2]	9.9 (9.4,10.5)	10.7 (9.6,11.9)	0.24	10.9 (10.2,11.8)	9.9 (9.3,10.4)	**0.03**
BMI (kg/m2)[1]	28.9 (28.3,29.5)	28.8 (27.6,30.1)	0.92	29.4 (28.6,30.2)	28.4 (27.8,29.0)	0.06
Body fat %[1]	33.5 (32.8,34.3)	34.3 (32.7,35.8)	0.41	33.8 (32.8,34.9)	33.8 (33.0,34.6)	0.93
Fasting insulin (pmol/L)[2]	59.4 (56.3,62.7)	57.5 (51.2,64.6)	0.62	56.0 (51.9,60.4)	59.6 (56.3,63.2)	0.20
Fasting glucose (mmol/L)	5.7 (5.6,5.8)	5.8 (5.6,5.9)	0.45	5.7 (5.5,5.8)	5.7 (5.6,5.8)	0.52
Insulin sensitivity IVGTT ((mU/L)$^{-1}$ min^{-1})[2]	2.8 (2.6,2.9)	2.6 (2.3,3.0)	0.51	2.9 (2.7,3.2)	2.7 (2.5,2.9)	0.22
HOMA2-IR[2]	2.2 (2.0,2.3)	2.1 (1.8,2.4)	0.72	2.0 (1.8,2.2)	2.2 (2.1,2.4)	0.11
NEFA (μmol/L)	695.8 (666.4,725.2)	667.6 (604.2,731.0)	0.43	686.3 (644.6,728.1)	701.1 (669.4,732.8)	0.58
TC (mmol/L)	5.7 (5.6,5.8)	5.6 (5.4,5.9)	0.72	5.7 (5.6,5.9)	5.7 (5.6,5.8)	0.65
TAG (mmol/L)[2]	1.5 (1.5,1.6)	1.5 (1.3,1.7)	0.86	1.5 (1.4,1.6)	1.5 (1.5,1.6)	0.30
HDL-C (mmol/L)[2]	1.4 (1.4,1.4)	1.4 (1.3,1.5)	0.92	1.4 (1.4,1.5)	1.4 (1.4,1.4)	0.37
Apo A1 (g/L)	1.2 (1.2,1.3)	1.2 (1.1,1.3)	0.48	1.3 (1.2,1.3)	1.2 (1.2,1.3)	0.25
LDL-C (mmol/L)	3.6 (3.5,3.7)	3.5 (3.3,3.7)	0.68	3.6 (3.5,3.8)	3.6 (3.5,3.7)	0.55
Apo B (g/L)	0.99 (0.96,1.03)	0.92 (0.84,0.99)	0.08	1.00 (0.95,1.05)	0.97 (0.94,1.01)	0.40

Data is presented for White European subjects for whom DNA samples were available (n = 366). Mean (95% CI) values stratified by genotype are shown for each SNP. Association was tested by ANCOVA based on a dominant model. [2]*P*-values derived from the GM values at baseline are presented. *P*-values in bold when nominally significant (< 0.05), adjusted for BMI, age and gender, [1]*P*-values are adjusted for gender and age.

Table 3.5 Serum adiponectin concentration, obesity measures, insulin homeostatic variables and lipid profile by *ADIPOQ* -7734 C/A and +276 G/T genotypes in White European

	-7734 C/A			+276 G/T		
Phenotype	CC	CA+AA	*P*	GG	GT+TT	*P*
Male/female	128/152	3/9		87/109	55/89	
Adiponectin (μg/mL)[2]	10.4 (9.9,10.9)	10.8 (8.5,13.8)	0.73	9.6 (9.1,10.2)	11.0 (10.2,11.8)	**0.006**
BMI (kg/m2)[1]	28.7 (28.2,29.3)	28.8 (26.1,31.5)	0.96	28.9 (28.2,29.6)	28.5 (27.7,29.3)	0.48
Body fat %[1]	33.5 (32.8,34.2)	33.4 (30.1,36.8)	0.98	34.2 (33.3,35.0)	33.3 (32.3,34.3)	0.18
Fasting insulin (pmol/L)[2]	58.4 (55.4,61.5)	54.2 (42.2,69.6)	0.57	60.9 (57.3,64.8)	55.6 (51.7,59.8)	0.06
Fasting glucose (mmol/L)	5.7 (5.6,5.8)	5.7 (5.2,6.1)	0.86	5.7 (5.6,5.8)	5.7 (5.5,5.8)	0.36
Insulin sensitivity IVGTT)((mU/L)-^{1} min-^{1})[2]	2.8 (2.6,3.0)	2.5 (1.8,3.3)	0.45	2.7 (2.5,2.9)	2.9 (2.6,3.1)	0.17
HOMA2-IR[2]	1.3 (1.2,1.4)	1.1 (0.9,1.4)	0.28	1.3 (1.3,1.4)	1.2 (1.1,1.3)	0.07
NEFA (μmol/L)	693.2 (665.4,721.1)	685.0 (549.9,820.0)	0.91	701.8 (667.2,736.4)	694.5 (654.1,734.9)	0.79
TC (mmol/L)	5.7 (5.6,5.8)	6.1 (5.5,6.6)	0.21	5.7 (5.6,5.8)	5.6 (5.5,5.8)	0.31
TAG (mmol/L)[2]	1.5 (1.4,1.6)	1.8 (1.5,2.2)	0.08	1.6 (1.5,1.7)	1.5 (1.3,1.6)	0.13
HDL-C (mmol/L)[2]	1.4 (1.4,1.4)	1.4 (1.3,1.6)	0.80	1.4 (1.4,1.4)	1.4 (1.4,1.5)	0.87
Apo A1 (g/L)	1.2 (1.2,1.3)	1.3 (1.2,1.4)	0.36	1.2 (1.2,1.3)	1.2 (1.2,1.3)	0.46
LDL-C (mmol/L)	3.6 (3.5,3.7)	3.8 (3.4,4.3)	0.44	3.6 (3.5,3.7)	3.5 (3.4,3.7)	0.46
Apo B (g/L)	0.99 (0.96,1.03)	1.15 (0.98,1.32)	0.07	0.98 (0.94,1.03)	0.97 (0.92,1.01)	0.59

Data is presented for White European subjects for whom DNA samples were available (n = 366). Mean (95% CI) values stratified by genotype are shown for each SNP. Association was tested by ANCOVA based on a dominant model. *P*-values derived from the GM values at baseline are presented. *P*-values in bold when nominally significant (< 0.05), adjusted for BMI, age and gender, [1]*P*-values are adjusted for gender and age.

3.3.7 Change in measured variables after dietary intervention

The RISCK study was designed to address the effects of diets with different macronutrient composition but similar energy intake, in order to measure changes in the absence of significant alteration in weight. Subjects were randomly assigned to continuation on the HS reference diet, or 24 weeks on an isoenergetic diet in which saturated fat was replaced with either HM diet or carbohydrate (LF diet). Diet during the run-in was monitored by weighed intake. There was no significant difference in intake of saturated fat between subjects who continued on the HS reference diet or were later assigned to the HM and LF diets (Moore et al, 2009). Body weight was relatively stable. Further information is provided elsewhere (Jebb et al, 2010; Moore et al, 2009). The changes in % SF and % MUFA differed between the diets over the 24 weeks of intervention. The HM group had significantly lower plasma phospholipid % SF than the LF group and higher % MUFA, but other fatty acid classes [(n-3) PUFA, (n-6) PUFA and trans FA] were not affected (Moore et al, 2009). Mean serum adiponectin concentration was not significantly different between the diet groups: after reference HS diet (n = 85) mean concentration = 9.2 (95% CI: 8.2, 10.3) μg/mL, after HM diet (n = 227) mean concentration = 9.3 (95% CI: 8.7, 10.0) μg/mL and after LF diet (n = 235) mean concentration = 9.6 (95% CI: 9.0, 10.2) μg/mL, with P = 0.44 after adjustment for BMI, age, gender and ethnicity. Dietary interventions had no significant effect on Si, unaltered after adjustment for change in weight. TC and LDL-C concentrations were significantly lower with the HM and LF than the HS diet (P < 0.001 and P < 0.001). Apo B concentrations differed between treatment groups (P < 0.001) and were lower with the HM and LF diets than with the HS diet. HDL-C concentrations were lower with the LF than with the HS or HM diets (P < 0.001 and P = 0.002, respectively). There were no significant changes in concentration of plasma TAG following interventions (Jebb et al, 2010).

3.3.8 Change in adiponectin concentrations after dietary intervention with respect to *ADIPOQ* SNP genotype

-10066 G/A and +276 G/T genotypes were significantly associated with serum adiponectin concentration at baseline in White subjects (**Tables 3.4 and 3.5**). We then investigated the significance of any changes in adiponectin concentration after the HM and LF diets, with respect to -10066 G/A, +276 G/T, -11391 G/A and -7734 C/A genotypes **Table 3.6**. There was no significant difference in change after HM or LF

diets with respect to +276 G/T, -11391 G/A or -7734 C/A genotypes, and none after the LF diet with respect to -10066 G/A genotype. However, after the HM diet there was a significant difference in change in serum adiponectin concentration between -10066 G/A genotype groups. Geometric mean concentration (95% CI) at baseline in G/G subjects (n = 57) was 10.4 (9.3, 11.6) μg/mL and in G/A+A/A (n = 94) 9.6 (8.8, 10.4) μg/mL. Geometric mean adiponectin concentration (95% CI) at follow up in G/G subjects was 10.8 (9.7, 12.1) μg/mL and in G/A+A/A was 9.3 (8.6, 10.2) μg/mL. -10066 G/G subjects showed an increase of 3.8 (-0.1, 7.7) % and G/A+A/A subjects a decrease of -2.6 (-5.6, 0.4) % after the HM diet. The difference in % change between G/G homozygotes and carriers of the A-allele was significant (P = 0.006) after adjustment for change in BMI, age and gender. However, gene x diet interaction in determination of serum adiponectin was not significant (P = 0.12) after adjustment for change in BMI, age and gender. We then analysed the data by excluding the subjects homozygous for the minor allele (n = 14), to ensure that they did not skew results when added to the heterozygote group. The interaction between -10066 G/A and diet remained insignificant. However, GG subjects still demonstrated significantly higher serum adiponectin concentration after HM, compared with the GA subjects (P = 0.01).

We also investigated possible -10066 G/A x diet interaction in determination of BMI, body fat %, fasting insulin, fasting glucose, Si, HOMA2-IR, NEFA, TC, TAG, HDL-C, Apo A1, LDL-C and Apo B were not significant (P >0.05) after adjustment for change in BMI, age and gender. Similarly no significant interaction between diet and +276 G/T , -11391 G/A or -7734 C/A was found in determining any of the phenotypes (P >0.05).

Table 3.6 Effect of high-MUFA and low-fat diets on adiponectin concentration with respect to *ADIPOQ* -11391 G/A, -10666 G/A, -7734 C/A and +276 G/T SNP genotypes in white European subjects

	HM				LF				
	Baseline	Follow-up	Percentage change		Baseline	Follow-up	Percentage change		diet x gene
Adiponectin (μg/ml)	GM (95%CI)	GM (95%CI)	GM (95%CI)	*P*	GM (95%CI)	GM (95%CI)	GM (95%CI)	*P*	*P*
10066 GG	10.4 (9.3,11.6)	10.8 (9.7,12.1)	3.8 (-0.1,7.7)	**0.006**	11.7 (10.4,13.1)	11.6 (10.3,13.1)	-0.5 (-4.8,3.8)	0.857	0.12
10066 GA+AA	9.6 (8.8,10.4)	9.4 (8.6,10.2)	-2.6 (-5.6,0.4)		9.9 (9.1,10.9)	9.8 (9.0,10.7)	-1.1 (-4.2,2.1)		
276 GG	9.1 (8.4,10.0)	9.1 (8.4,10.0)	-0.1 (-3.3,3.0)	0.841	9.9 (8.9,11.0)	10.1 (9.1,11.2)	1.7 (-1.9,5.4)	0.107	0.24
276 GT+TT	11.2 (10.1,12.5)	11.3 (10.1,12.6)	0.4 (-3.6,4.4)		10.7 (9.6,12.0)	10.5 (9.4,11.7)	-2.5 (-6.2,1.2)		
7734 CC	10.1 (9.4,10.8)	9.9 (9.2,10.7)	-1.4 (-4.3,1.5)	0.51	10.7 (9.8,11.6)	10.5 (9.7,11.4)	-1.5 (-4.2,1.3)	0.11	0.07
7734 CA+AA	10.2 (7.9,13.4)	9.7 (7.4,12.9)	-5.2 (-16.2,5.7)		11.7 (7.4,18.5)	13.2 (8.3,21.0)	11.3 (-4.2,26.9)		
11391 GG	9.8 (9.1,10.5)	9.7 (9.0,10.4)	-0.9 (-3.8,1.9)	0.22	9.9 (9.1,10.8)	9.9 (9.1,10.8)	-0.3 (-3.2,2.5)	0.95	0.51
11391 GA+AA	10.1 (8.6,11.8)	10.4 (8.9,12.1)	3.3 (-2.8,9.3)		11.6 (9.6,14.1)	11.6 (9.6,14.0)	-0.6 (-6.9,5.8)		

Mean baseline, follow-up and percentage change in GM of adiponectin (95% CI) values stratified by genotype and diet (HM or LF) are shown for each SNP. Association was tested by ANCOVA on a dominant model. *P*-values derived from the % change in GM of adiponectin. *P*-values in bold when nominally significant (< 0.05), adjusted for change in BMI, age and gender.

3.3.9 Change in adiponectin concentrations after dietary intervention with respect to age and -10066 G/A genotype

We were interested to determine whether the significant increase in serum adiponectin concentration with age that we observed at baseline **(Table 3.2)** was modified by dietary intervention. After dietary treatment, interaction between diet x age was not significant in determining the change in concentration ($P = 0.83$) adjusted for change in BMI and gender. We then proceeded to determine whether age and -10066 G/A genotype interacted to influence the change in adiponectin concentrations after dietary intervention. **Figure 3.3** compares the effect of HM and LF diets on % change in serum adiponectin concentration in White -10066 G/G homozygotes and A-allele carriers. Although there were inconsistent effects in the smallest number of subjects in the 35-40 years age group ($n = 41$), general trends were evident across the 10 years categories from age 41-70 years. In G/G homozygotes over 40 years of age, adiponectin concentration increased progressively after the HM diet, and decreased after the LF diet (**Figure 3.3A**). The difference in % change in serum adiponectin between G/G subjects on HM and LF diets in the oldest 61-70 years age group was significant ($P = 0.003$). In A-allele carriers there was little change in serum adiponectin concentration compared to baseline with increasing age, after HM or LF diets (**Figure 3.3B**). Interaction between gene x age x diet in determination of change in serum adiponectin concentration approached significance after adjustment for gender and change in BMI ($n = 303$; $P = 0.07$) and remained insignificant after correction for multiple testing. However, the interaction between gene x age x diet x gender was not significant ($P = 0.67$) after adjustment for change in BMI.

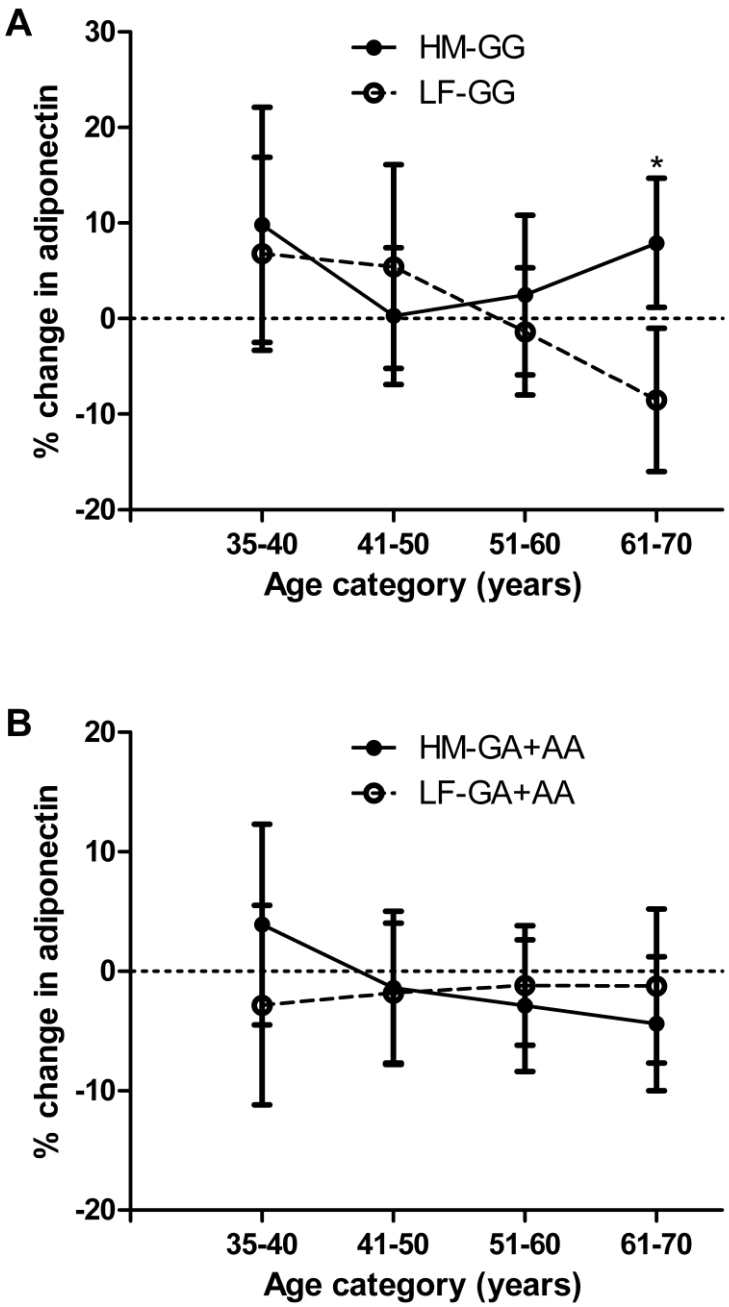

Figure 3.3 Effect of HM and LF diets on adiponectin concentration with respect to -10066 G/A genotype and age in White subjects. % changes (95% CI) in GM adiponectin concentration adjusted for change in BMI and gender are shown in each age group, after subjects consumed HM (*n* = 151) or LF (*n* = 152) diets. (A) -10066 G/G subjects (*n* = 111) and (B) -10066 G/A+A/A subjects (*n* = 192). Interaction between gene x age x diet in determination of change in serum adiponectin concentration determined by ANCOVA, was not significant after adjustment for change in BMI and gender (*n* = 303; *P* = 0.07). *Denotes significant difference in % change in serum adiponectin between G/G subjects on HM and LF diets (*P* = 0.003). (A) -10066 G/G subjects; age 30-40, 41-50, 51-60 and 61-70 y on HM diet (*n* = 6, 18, 13 and 20 respectively) and LF diet (*n* =9, 8, 21 and 16 respectively). (B) -10066 G/A+A/A subjects; 30-40, 41-50, 51-60 and 61-70 y on HM diet (*n* = 13, 22, 30 and 29 respectively) and LF diet (*n* = 13, 27, 36 and 22 respectively).

3.4 Discussion

In this cohort of mainly overweight men and women, we have shown significant effects of age, BMI and ethnicity on serum adiponectin concentration. We have demonstrated a novel influence of *ADIPOQ* -10066 G/A genotype dependent on age and diet. After the HM diet, G/G homozygotes showed a progressive increase in serum adiponectin concentrations between 41-70 years of age, whereas after the LF diet there was a progressive decline.

3.4.1 Influence of body mass index, ethnicity, age and gender

The significant increase in serum adiponectin concentration that we found with age, after adjustment for BMI, is in agreement with previous reports (Adamczak et al, 2005). As insulin sensitivity declines with age, this may reflect development of resistance or survival in those with higher concentrations. The inverse relationship that we found with BMI is well known (Isobe et al, 2005). Serum adiponectin was found to be significantly higher in White Europeans than in S. Asians and Black Africans after adjustments for age, BMI and gender. Similarly, Cohen et al. (2011) reported that Black subjects had significantly lower adiponectin concentrations than White subjects. They also reported inconsistent correlations of adiponectin with BMI and age in White and Black women (Cohen et al, 2011). Moreover, Hulver et al. (2004) have suggested that BMI, insulin, and HOMA correlated significantly with adiponectin concentrations only in Caucasian women but not in African-American women. These studies suggest that relationships with obesity and insulin sensitivity may not be generalisable to all ethnic groups. We focused our genetic investigation on the White Europeans. We also found significant, substantially higher serum adiponectin in women compared to men, as reported previously (Marques-Vidal et al, 2010) and those differences persisted after adjusting for age or fat mass. This was contrary to some studies that reported no gender differences in adiponectin concentration (Kuo & Halpern, 2011). Our data indicate that gender differences in adiponectin cannot be accounted for solely by differences in body fat between genders. Other factors might thus intervene, such as diet (Pischon et al, 2005) or physical activity levels (Tsukinoki et al, 2005).

3.4.2 Correlation between adiponectin and metabolic risk factors

We found a significant negative correlation between adiponectin and BMI, HOMA2-IR, and TAG. This is in line with the findings of other studies (Isobe et al, 2005; Brochu-Gaudreau et al, 2010; Hotta et al, 2000; Meilleur et al, 2010; Cassidy et al, 2009). Others reported a negative correlation between adiponectin and BMI, insulin and HOMA (Hulver et al, 2004). We also found a positive correlation with Si, body fat %, HDL-C, Apo A1 and TC. Similarly, Park et al (2010) reported a significant association with HDL-C and suggested that in postmenopausal women, this may have a protective effect on atherosclerosis. A positive correlation with HDL-C and TC after adjusting for age, gender and BMI was found in West African populations (Meilleur et al, 2010).

3.4.3 SNP associations at baseline

Previously in our laboratory, Kyriakou et al. (2008) observed strong associations of the four SNPs with serum adiponectin concentrations in two much larger cohorts of healthy White women. In White European subjects from the RISCK study, at risk of development of the metabolic syndrome we found significantly higher mean serum adiponectin at baseline in variant +276 T-allele carriers than non-carriers. In agreement with our findings, association of elevated adiponectin with +276T has been reported in several studies (Menzaghi et al, 2002; Hara et al, 2002). We found that carriage of the variant -10066 A-allele was associated with significantly lower serum adiponectin at baseline than non-carriers; this remained significant after correction for multiple comparisons, in agreement with previous reports (Woo et al, 2006; Kyriakou et al, 2008). The remainder of our study centres on this SNP. Although the association of elevated adiponectin with the -11391 A-allele has been reported widely (Vasseur et al, 2002; Woo et al, 2006; Heid et al, 2006; Bouatia-Naji et al, 2006), in our study population -11391, A-allele has a tendency towards higher adiponectin. However, the difference did not reach significance levels (P=0.24). While another group found lower adiponectin in-11391 G-allele carriers (Petrone et al, 2006). Furthermore, there are several reports of increased risk of insulin resistance and T2D associated with the -11391 GG genotype (Vasseur et al, 2002; Petrone et al, 2006). We found the same trends in RISCK -11391 GG White subjects with higher fasting insulin and HOMA2-IR but greater insulin sensitivity measured by Si. However, these associations did not reach significant levels. We did not replicate the previous association of the -7734 A-allele with elevated adiponectin (Kyriakou et al, 2008) in the RISCK subjects; however we

found a trend for 7734 A-allele carriers to have elevated adiponectin. Some studies reported associations between *ADIPOQ* SNPs, BMI and blood lipids. For example, +276G was associated with higher BMI in Italian subjects (Menzaghi et al, 2002) but with lower BMI in Swedish and African Americans (Ukkola et al, 2003; Beebe-Dimmer et al, 2010) and higher concentration of TAG (Jang et al, 2005). We found no association between *ADIPOQ* SNPs and measures of obesity or lipid profile in the RISCK subjects.

3.4.4 Dietary intervention

Epidemiological evidence and intervention studies strongly suggest that the quality of dietary fat influences insulin sensitivity in humans (Vessby et al, 2001). However, in the full RISCK cohort, replacement of SFA by isoenergetic MUFA or carbohydrate diets for 24 weeks did not significantly improve adiponectin concentration. Previously Jebb et al. (2010) reported no significant effect on insulin sensitivity following this dietary regimen. Small changes in adiponectin concentration after dietary intervention may not have been sufficient to affect insulin sensitivity, or the intervention period may not have been long enough to produce an effect. This is consistent with other reports, where no association of total energy or macronutrient intake with serum adiponectin is evident in young subjects (Yannakoulia et al, 2003), or on the impact of hypocaloric diets on adiponectin concentrations in obese women (Arvidsson et al, 2004), or else following a high-fat, low-carbohydrate meal (Peake et al, 2003). Long term effects were seen only after a 10 year Mediterranean diet in diabetic women (Mantzoros et al, 2006) and postmenopausal obese women (Esposito et al, 2003). These data suggest that adiponectin concentrations are unlikely to be affected by relatively short term dietary changes, but rather reflect intakes over longer time periods (Pischon et al, 2005).

3.4.5 SNP associations after dietary intervention

In a recent study, an interaction between *ADIPOQ* rs266729 (-11377 C/G) genotype with SFA, but not MUFA or PUFA, significantly affected HOMA-IR. However, there were no significant effects on serum adiponectin concentration, suggesting that insulin resistance did not reflect changes in *ADIPOQ* gene expression elicited by the diet (Ferguson et al, 2010). We hypothesised that stratification by genotype might uncover influential interaction between diet and *ADIPOQ* variants. There were no significant

differences in the change in serum adiponectin concentration after HM or LF diets with respect to +276 G/T, 11391 G/A or -7734 C/A genotypes and none after the LF diet with respect to -10066 G/A. However, after the HM diet, G/G subjects showed an increase of 3.8 % and G/A+A/A subjects a decrease of 2.6%. Although the difference in change was highly significant, gene x diet interaction was not a significant determinant of serum adiponectin. Activation of PPARγ by unsaturated fatty acids increases with chain length and degree of unsaturation (Sanderson et al, 2008). The switch from SFA to MUFA could lead to increased expression of the *ADIPOQ* gene and serum adiponectin concentration through increased availability of PPARγ-activating ligands. The PPRE is 250 bp upstream of exon 1 (Iwaki et al, 2003) and lies in a 1.3 kb LD block bounded 5' by -11377 G/C in the promoter and 3' by -10066 G/A in intron 1 (Heid et al, 2006). If the rare -10066 A-allele was in linkage disequilibrium with a variant in the PPRE, reducing affinity for the receptor, this could account for higher serum adiponectin in response to MUFA in G/G homozygotes, and the lower concentration in A-allele carriers. It is worth noting that -11391 G/A, which was not associated with adiponectin concentration in RISCK subjects, lies upstream of the LD block containing the PPRE (Heid et al, 2006).

3.4.6 SNP associations after dietary intervention and interaction with age

We had previously shown that adiponectin increased with age in males and females, but age did not interact with +276 G/T or -10066 G/A genotypes in determining adiponectin concentrations in White subjects at baseline. We were interested to discover whether the strong relationship between adiponectin concentration and age seen at baseline was modified by diet. We looked at whether age x genotype interaction was influential after dietary intervention.

After the HM diet, -10066 G/G homozygotes showed a progressive increase in serum adiponectin between 41-70 years of age, culminating in a highly significant difference in the oldest age group. However, after the LF diet, G/G homozygotes showed a progressive fall. Both diets produced little effect in A-allele carriers. Serum adiponectin might be expected to be lower in G/G subjects after the LF diet, in which carbohydrate replaces SFA, than after the HM diet. In A-allele carriers, substitution of SFA for MUFA or carbohydrate would have little effect if reduced affinity of the PPRE, rather than ligand activation were to be the rate-limiting step. This scenario would be

compatible with other reports of lower serum adiponectin after high carbohydrate (Pischon et al, 2005; Esposito et al, 2003) and higher serum adiponectin with a diet rich in MUFA, compared with a carbohydrate or protein rich diet (Yeung et al, 2010). If aging is associated with the development of adiponectin resistance, the change in adiponectin concentrations may reflect a capability of responding by increasing production after HM, but not LF, diets.

Few studies have explored the relationship between dietary factors and adiponectin concentrations or gene-nutrient interactions involving SNPs at the *ADIPOQ* locus. In a larger study (n = 1083), there was no interaction between dietary fat and -11391 G/A genotype in determination of serum adiponectin concentration, but A-allele carriers had lower BMI than non-carriers when MUFA comprised >13% total energy intake (Warodomwichit et al, 2009). Nelson et al. (2007) have indicated reduced adiponectin concentrations after α-linolenic acid supplementation in overweight individuals (n = 57) independent of two *ADIPOQ* SNPs (+276 G/T and +45 T/G), which have no effect. Perez-Martinez et al. (2008) have studied 59 healthy young men and women on dietary regimes similar to RISCK: an initially SFA-enriched diet, followed by a MUFA-rich or carbohydrate-rich (LF) diet for 28 days in a randomised, crossover design. They found no interaction between another *ADIPOQ* promoter, SNP –11377 C/G, and diet-affecting plasma adiponectin concentration in all subjects, but male C/C homozygotes had lower adiponectin than C/C women independently of the dietary fat. C/C homozygous men were less insulin after MUFA-rich and low-fat diets, than after SFA-rich diet. SNPs residing outside the coding region of the gene may have functional significance, or simply be linkage disequilibrium markers.

3.5 Limitations and conclusion

Limitations include a relatively small sample size and changes in serum adiponectin concentration. We measured total adiponectin, rather than the most bioactive HMW form, but a strong correlation has been shown between the two measures, regardless of obesity status or dietary period (Yeung et al, 2010). Wide inter-individual variation at baseline and in response to diets could have limited the significance of some outcomes. Replication in other cohorts is the most reliable method to distinguish true from false-positive associations. If substantiated in a larger sample, a recommendation to -10066 G/G homozygotes to substitute SFA with MUFA to maintain adiponectin

concentrations with advancing years would be justified. Elucidation of the effects of common SNPs in modifying the outcome of dietary intervention studies should help in the identification of individuals at risk of complex disease who would benefit from personalized dietary recommendations.

Chapter 4

The Influence of PPAR-γ2 Gene Pro12Ala SNP and Dietary Intake of Fat on Adiponectin Concentrations and Plasma Lipids

4.1 Introduction

PPARγ is one of three PPARs and a member of the nuclear hormone receptor superfamily (Desvergne & Wahli, 1999). The major natural ligands are PUFAs and prostanoids (Xu et al, 1999), suggesting a role in transducing nutritional to metabolic signals (Semple et al, 2006). An increase in PPARγ mRNA in adipose tissue of mice exposed to a high fat diet (Vidal-Puig et al, 1996) suggested that dietary modulation might influence adipogenesis induced by PPARγ in response to raised plasma concentration of fatty acid ligands.

Variants of the PPARγ2 gene *PPARG* could alter transcriptional activity of the activator through DNA- and/or ligand-binding affinity. Previous studies have investigated *PPARG* Pro12Ala genotype associations with risk of obesity and diabetes, with equivocal results (Altshuler et al, 2000; Tonjes et al, 2006; Masud & Ye, 2003) suggesting that environmental influences such as dietary intake may be involved. Luan et al. (2001) found BMI and fasting insulin in Ala12-allele carriers but not Pro/Pro homozygotes, was influenced by ratio of habitual dietary P:S intake. Others showed a relationship between intake of total fat and BMI (Memisoglu et al, 2003) or waist circumference (Robitaille et al, 2003) in common Pro/Pro homozygotes, but not in Ala12-allele carriers. However, when MUFA rather than total fat was examined, intake was found to be inversely associated with BMI in Ala12-allele carriers and not in Pro/Pro homozygotes (Memisoglu et al, 2003). *In vitro*, the PPARγ2 Ala-variant exhibits reduced binding to DNA and modest impairment of transcriptional activation following treatment with pharmacological ligand TZDs (Deeb et al, 1998, Masugi et al, 2000). This raised the possibility that differential responses by Pro/Pro homozygotes and Ala12-allele carriers to unsaturated fatty acid ligands of PPARγ might influence adipogenesis.

PPARγ target genes include several involved in adipogenesis (Tontonoz & Spiegelman, 1994), insulin sensitivity (Gurnell, 2003) and lipid metabolism (Walczak & Tontonoz, 2002) We hypothesised that dietary intake of unsaturated fats might interact with *PPARG* Pro12Ala genotype to influence concentration of adiponectin, plasma lipids, measures of obesity and insulin sensitivity.

We aimed to test

1- The association of genotype with plasma lipids, BMI, waist circumference, fasting insulin, and glucose and HOMA-IR at recruitment screening (habitual diet) and after 4 weeks on a HS diet in White subjects at risk of the metabolic syndrome.
2- Whether habitual P: S ratio modulates the effect of *PPARG* Pro12Ala on markers of metabolic syndrome.
3- Interaction between genotype and dietary fat intake after the intervention in determining mean quantitative phenotypes.

4.2 Subjects and Methods

4.2.1 Subjects

This work was based on the participants of the RISCK study. Refer to **Section 2.1.1** for details of RISCK study participants.

4.2.2 Study design

Refer to **Section 2.1.1** for description of RISCK study design.

4.2.3 Blood analytic methods and anthropometry measurements:

For serum adiponectin, blood lipids, insulin sensitivity and anthropometric measurements; refer to **Sections 2.3.1.1, 2.3.1.2, 2.3.1.3 and 2.3.2** respectively**.**

4.2.4 DNA extraction and SNP genotyping

Details on methods for DNA extraction from buffy coats are given in **Section 2.3.4**. The *PPARG* Pro12Ala SNP was genotyped by KBiosciences (Hoddesdon, UK) for 466 subjects who had consented to genetic analysis using the Kaspar system. Genotype accuracy as assessed by inclusion of duplicates in the array was 98% and negative controls (water blanks) were included on each plate. Genotyping success rate was 89%.

4.2.5 Statistical analyses

PPARG Pro12Ala genotype distributions were tested for deviation from the Hardy-Weinberg equilibrium by a χ^2 test with 1 df ($P > 0.05$). Statistical analyses were carried out using the SPSS version 17.0 for Windows (SPSS Inc., Chicago, IL, USA). Values for Si, HOMA2-IR, HOMA-IR, insulin, adiponectin, TAG and HDL-C were log transformed to obtain better approximations of the normal distribution prior to analysis. Data were analysed by ANCOVA with BMI, age, gender and diet as covariates. Outliers were excluded from the ANCOVA and were defined as points >2.5 times the interquartile range from the median on the transformed scale at recruitment or after HS diet. All data presented in text and tables are expressed as means or geometric means ± SD or 95% CI. Statistical significance was taken at $P < 0.05$. ANCOVA was used to test the interaction between genotypes and habitual P:S quartiles or intervention diets. In

this model, the dependant variable was the analysed plasma lipid (TAG, LDL-C or TC) and fixed factors were the genotypes and P:S quartiles or dietary intake of fat (HS,LF and HM). BMI, age and gender were the covariates. Figures were drawn using Prism version 5 (GraphPad software, Inc., California, and USA).

4.3. Results

4.3.1 Characteristics of subjects

Refer to **Section 3.3.1** and **Table 3.1** for the characteristics of subjects who completed the study. The 466 individuals for whom DNA samples were available are the subjects of this study. Their characteristics at baseline with respect to ethnicity, age, obesity measures, insulin homeostatic variables, adiponectin, lipid profile and BP are presented in **Table 4.1**. After 4-week run-in on the HS diet, there were significant differences between males and females in fasting glucose and insulin, waist circumference, TAG, systolic and diastolic BP (higher in males), HDL-C, adiponectin, Si and body fat % (lower in males).

Table 4.1 Characteristics of RISCK study subjects at baseline

Phenotype	All (n = 466)	Males (n = 191)	Females (n = 275)	p
Ethnicity[1]				
South Asia (n)	44	19	25	
Black African (n)	38	10	28	
White European (n)	367	155	212	
Others	17	7	10	
Age (years)	52.4± 9.9	53.5± 10.3	51.6± 9.5	0.042
Height (m)	1.7± 0.1	1.8± 0.1	1.6± 0.1	<0.001
Weight (kg)	81.8± 15.7	88.8± 13.1	77.0± 15.5	<0.001
BMI (kg/m^2)	28.7± 4.7	28.6± 3.9	28.8± 5.2	0.559
Waist circumference (cm)	98.0± 12.3	103.0± 10.4	94.7± 12.4	<0.001
Body fat (%)	34.0± 8.4	26.7± 5.1	39.0± 6.3	<0.001
Systolic BP (mm Hg)	129.5± 15.9	135.1± 15.2	125.6± 15.3	<0.001
Diastolic BP (mm Hg)	79.5± 9.5	83.0± 9.0	77.1± 9.1	<0.001
Fasting insulin (pmol/L)[2]	61.2± 246.6	65.8 ± 378.7	58.1± 57.8	0.024
Fasting glucose (mmol/L)	5.7± 0.8	5.9± 0.9	5.5± 0.6	<0.001
Insulin sensitivity (IVGTT)((mU/L)-1 min-1)[2]	2.6 ± 5.8	2.2± 1.9	2.9± 7.3	<0.001
HOMA2-IR[2]	1.3± 0.7	1.3± 0.8	1.3± 0.7	0.451
TAG (mmol/L)[2]	1.5± 0.7	1.7± 0.8	1.4± 0.6	<0.001
TC (mmol/L)	5.6± 1.0	5.6± 1.0	5.6± 1.0	0.57
HDL-C (mmol/L)[2]	1.4± 0.3	1.3± 0.3	1.5± 0.3	<0.001
Apo A1 (mg/dL)	1.2± 0.3	1.2± 0.2	1.3± 0.2	<0.001
LDL-C (mmol/L)	3.5± 0.8	3.6± 0.8	3.5± 0.8	0.143
Apo B (mg/dL)	0.97± 0.3	1.00± 0.3	0.955± 0.3	0.089
Adiponectin (μg/mL)[2]	9.5± 5.9	7.7± 4.2	11.1± 6.4	<0.001

Data is presented for subjects which DNA samples were available (n = 466). Mean ± SD, or [2]GM ± SD. All variables were measured at baseline after 4-week run-in on reference HS diet. [1]Self-reported ethnicity. Significance of differences between women and men was determined by T-test.

In view of the small sample size of the S. Asian and other ancestries and absence of the *PPARG* Pro12Ala in Blacks, we chose to focus our genetic investigation on the White subjects only. The characteristics at recruitment of the White participants (n = 367) who completed the study and for whom DNA was available are presented in **Table 4.2**

Table 4.2 Characteristics of White RISCK study subjects at recruitment screening

Phenotype	Male (n = 155)	Female (n = 212)
Age (years)	54±10	53±10
Waist circumference (cm)	103.1±10.7	95.4±12.6
BMI (kg/m^2)	28.6±4.0	29.1±5.3
TAG (mmol/L)[1]	1.4±0.8	1.2±0.7
TC (mmol/L)	5.6±0.8	5.7±1.0
LDL-C (mmol/L)	3.6±0.8	3.5±0.9
HDL-C (mmol/L)[1]	1.2±0.3	1.5±0.4

Data measured at recruitment is presented for all White subjects who completed the study and for whom DNA samples were available (n = 367). Values are mean or [1]GM ± SD.

4.3.2 *PPARG* Pro12Ala allele and genotype frequencies

All available DNA samples were genotyped (n = 466) and data was obtained for 415 subjects. **Table 4.3** shows the allele and genotype frequencies for *PPARG* Pro12Ala in subjects stratified by ethnic group. Genotype distributions did not deviate from Hardy-Weinberg expectations. The MAFs were similar to those listed on the NCBI SNP database (http://www.ncbi.nlm.nih.gov/snp; build 132 accessed 15/12/10). The Ala12-allele was more frequent than expected in White Europeans (0.10 in RISCK compared to 0.076 in HapMap-CEU (European)) and absent in Black Africans, as in Hap-Map trios (HapMap- YRI (Sub-Saharan African)). There are no comparative data available for S. Asians.

Table 4.3 *PPARG* Pro12Ala genotype allele and genotype frequencies

	Ethnic group[1]				
	S. Asian [n (%)] 44 (9)	Black African [n (%)] 38 (8)	White European [n (%)] 367 (79)	Other [n (%)] 17 (4)	All [n (%)] 466 (100)
MAF	0.07	0	0.1	0.1	0.09
Pro/Pro	36 (86)	36 (100)	258 (80)	12 (80)	342 (82)
Pro/Ala	6 (14)	0 (0)	61 (19)	3 (20)	70 (17)
Ala/Ala	0 (0)	0 (0)	3 (1)	0 (0)	3 (1)
Total	42 (100)	36 (100)	322 (100)	15 (100)	415 (100)

All subjects for whom DNA samples were available were genotyped (n = 466); n (%) is number of each ethnic group genotyped, as % of total. Total with genotype data (n = 415); n (%) refers to number of each genotype obtained, with % genotype frequency. [1]Self-reported ethnicity.

4.3.3 *PPARG* Pro12Ala genotype associations with phenotypes at recruitment screening

Table 4.4 shows anthropometry, plasma lipid concentrations, fasting glucose and insulin and HOMA-IR values in White subjects at recruitment (screening) with respect to *PPARG* Pro12Ala genotype. There were no significant associations between *PPARG* Pro12Ala genotype and these variables in White subjects at recruitment, after adjustment for BMI, gender and age ($P > 0.05$). Pro/Pro homozygotes had higher BMI, waist circumference and % of body fat compared to Ala12-allele carriers. However the differences did not reach a level of significance ($P > 0.05$).

Table 4.4 Associations between *PPARG* Pro12Ala genotypes and phenotypes at recruitment in White subjects

Phenotype	*PPARG* Pro12Ala genotype		*P*
	Pro/Pro	Pro/Ala + Ala/Ala	
Males n(%)	102(74%)	35(26%)	
Females n(%)	156(84%)	29(16%)	
BMI (kg/m^2)[1]	29.0 (28.4,29.5)	27.8 (26.7,29.0)	0.08
Waist circumference (cm)[1]	98.9 (97.4,100.4)	97.4 (94.3,100.5)	0.10
Body fat (%)[1]	34.5 (33.3,35.7)	31.4 (29.1,33.6)	0.24
Fasting insulin (pmol/L)[2]	58.6 (55.6,61.8)	54.6 (48.9,60.8)	0.46
Fasting glucose (mmol/L)	5.4 (5.3,5.5)	5.4 (5.3,5.6)	0.87
HOMA-IR[2]	2.2 (2.1,2.4)	2.0 (1.8,2.3)	0.43
TC (mmol/L)	5.6 (5.5,5.7)	5.7 (5.5,5.9)	0.42
TAG (mmol/L)[2]	1.3 (1.2,1.3)	1.3 (1.1,1.4)	0.96
HDL-C (mmol/L)[2]	1.4 (1.4,1.4)	1.4 (1.3,1.4)	0.54
LDL-C (mmol/L)	3.5 (3.4,3.6)	3.6 (3.4,3.8)	0.54

Data is presented for subjects for whom genotypic and phenotypic data was available ($n = 322$). Mean (95% CI), or [2]GM mean (95% CI) values, stratified by genotype are shown at recruitment. Association was tested by ANCOVA based on a dominant model. *P*-values adjusted for BMI, age and gender [1]*P*-values adjusted for gender and age.

4.3.4 Interaction between *PPARG* Pro12Ala genotype and habitual dietary P:S ratio

The full cohort was divided into quartiles of P:S, regardless of their genotypes. However, because of some missing genotyping results, when looking at the P:S interaction with Pro12Ala, the numbers of the subjects in each quartile were not equal. There was a significant interaction between dietary P:S ratio and genotype as determinants of plasma TC ($P = 0.02$), LDL-C ($P = 0.002$), and TAG ($P = 0.02$) concentrations after adjustment for BMI, age and gender in White subjects. **Table 4.5**

shows plasma TC, LDL-C and TAG concentrations with respect to genotype in quartiles of habitual P:S intake. When the P:S ratio was low (≤ 0.33), mean plasma TC concentration in Ala12-allele carriers was significantly higher than in non-carriers (P = 0.003). In the remaining quartiles, as P:S increased, the concentration of TC fell by 10 %. The trend in reduction as the ratio increased from ≤ 0.33 to >0.65 was significant (P = 0.02). The same pattern was seen in the concentration of LDL-C, with a significant difference in concentration between carriers and non-carriers in the lowest P:S quartile (P = 0.0001). As P:S increased in remaining quartiles, the concentration of LDL-C fell by 19.5% in Ala12-allele carriers, but here the trend was not significant (P > 0.05). There were no significant differences in plasma TAG concentrations between carriers and non-carriers of the Ala12-allele in any P:S quartile, however, there was a significant trend in reduction of plasma TAG concentration in Ala12-allele carriers as the P:S ratio increased from 0.34 to >0.65, in which concentration fell by 50.0% (P = 0.002). Plasma TAG concentration stratified by genotype and P:S quartile is shown in **Figure 4.1.** No significant interaction was found with dietary P:S ratio and genotype as determinants of BMI, waist circumference, body fat %, fasting insulin and glucose, HOMA-IR, or TC and HDL-C (P > 0.05). We then analysed the data by excluding the subjects homozygous for the minor allele (n = 3), to ensure they did not skew the results when added to the heterozygote subjects. The interaction between Pro12Ala and P:S remained significant for LDL-C, TC and TAG (P= 0.006, 0.04 and 0.03, respectively).

Table 4.5 Plasma lipid concentrations with respect to Pro12Ala genotype and quartiles of habitual dietary P:S intake ratio

	TC					LDL-C					TAG[1]				
P:S quartile	Pro/Pro Mean (95% CI)	*n*	Pro/Ala+Ala/Ala Mean (95% CI)	*n*	*P*	Pro/Pro Mean (95% CI)	*n*	Pro/Ala+Ala/Ala Mean (95% CI)	*n*	*P*	Pro/Pro Mean (95% CI)	*n*	Pro/Ala+Ala/Ala Mean (95% CI)	*n*	*P*
≤0.33	5.4 (5.2,5.6)	64	6.1 (5.7,6.6)	16	**0.003**	3.3 (3.1,3.5)	64	4.1 (3.7,4.6)	16	**0.0001**	1.3 (1.2,1.5)	64	1.1 (0.9,1.4)	16	0.09
0.34-0.47	5.7 (5.5,5.9)	76	5.8 (5.1,6.5)	8	0.92	3.6 (3.4,3.8)	76	3.3 (2.6,3.9)	9	0.16	1.3 (1.2,1.4)	76	2.0 (1.4,2.8)	8	0.09
0.48-0.65	5.6 (5.3,5.8)	59	5.5 (5.1,5.9)	19	0.71	3.5 (3.3,3.7)	59	3.3 (2.9,3.8)	19	0.51	1.2 (1.1,1.4)	59	1.4 (1.1,1.8)	19	0.19
>0.65	5.8 (5.5,6.0)	49	5.5 (5.0,6.0)	17	0.33	3.7 (3.4,3.9)	49	3.6 (3.1,4.0)	17	0.67	1.2 (1.0,1.3)	49	1.0 (0.8,1.3)	17	0.37

Data is presented for subjects for whom genotypic and phenotypic data was available (*n* = 322). Mean (95% CI), or [1]GM mean (95% CI) values stratified by genotype are shown after habitual diet. Association was tested by ANCOVA based on a dominant model. *P*-values adjusted for BMI, age and gender.

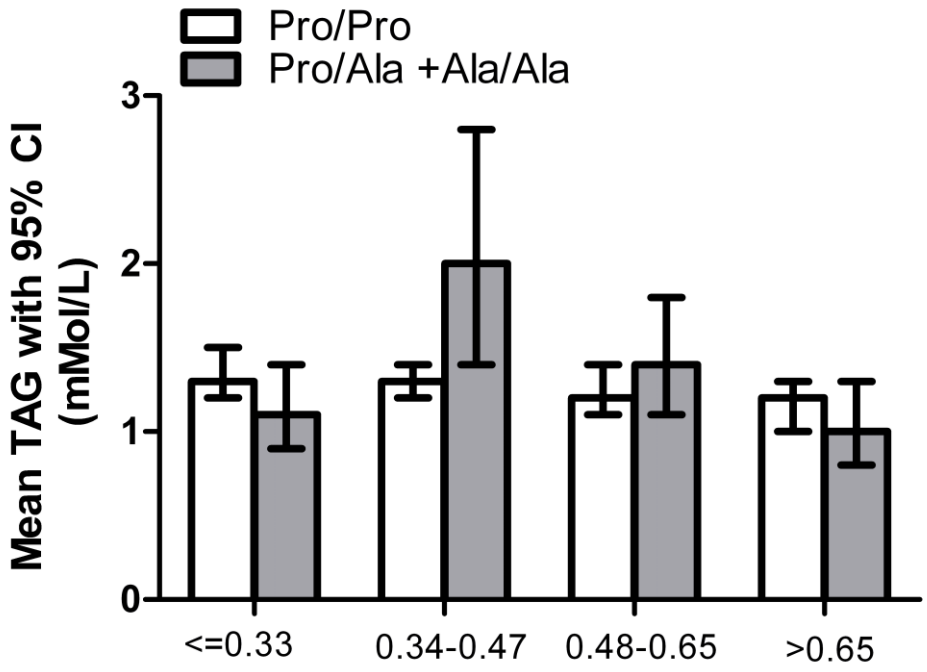

Figure 4.1 Mean TAG concentrations with respect to quartiles of habitual dietary P:S ratio and *PPARG* Pro12Ala genotype. The numbers of genotyped subjects with measurements in each quartile of P:S ratio were as shown in **Table 4.5**. GM concentration of TAG is shown. Bars represent 95% CI. Dietary P:S ratio x genotype interaction determined by ANCOVA significantly influenced plasma TAG concentration (P = 0.02), after adjustment for BMI, gender and age. There was a significant trend in reduction of plasma TAG concentration between P:S ratio 0.34 to >0.65 (P = 0.002) in Ala12-allele carriers.

4.3.5 Change in measured variables after dietary intervention

Details on changes on blood lipids, Si and adiponectin after dietary intervention can be found in **Section 3.3.7.**

4.3.6 *PPARG* Pro12Ala genotype associations with plasma lipid, adiponectin concentrations, insulin sensitivity and anthropometry measurement at baseline

Table 4.6 shows plasma lipids concentrations, obesity measures, insulin sensitivity measures and adiponectin concentrations with respect to *PPARG* Pro12Ala genotype after the 4-week HS diet at baseline in White subjects. No significant differences in these variables were found between the two genotypes. The HS diet consumed during the 4-week run-in to baseline comprised 18% of energy SFA. The P:S intake ratio in White subjects at baseline was 0.35 i.e. in the second quartile of habitual P:S intake (0.34 – 0.47). At baseline, carriers of the Ala12-allele (n = 64) had higher plasma concentrations compared to non-carriers (n = 258) of TC, LDL-C and Apo B, but differences were not significant after adjustment for BMI, gender and age.

Table 4.6 Associations between *PPARG* Pro12Ala genotypes and phenotypes at baseline in White subjects

	PPARG Pro12Ala genotype		
Phenotype	Pro/Pro	Pro/Ala + Ala/Ala	*P*
Males *n* (%)	102 (40%)	35 (55%)	
Females *n* (%)	156 (60%)	29 (45%)	
BMI (kg/m^2)[1]	28.9 (28.3,29.4)	27.8 (26.8,28.9)	0.13
Waist circumference (cm)[1]	98.4 (96.8,100.0)	97.6 (94.7,100.6)	0.23
Body fat %[1]	34.2 (33.2,35.3)	31.4 (29.3,33.4)	0.19
Insulin sensitivity (IVGTT)((mU/L)-1 min-1)[2]	2.7 (2.5,3.0)	2.8 (2.5,3.2)	0.81
Fasting insulin (pmol/L)[2]	59.0 (55.4,62.7)	54.4 (47.6,62.2)	0.49
Fasting glucose (mmol/L)	5.7 (5.6,5.7)	5.8 (5.5,6.1)	0.26
HOMA2-IR[2]	1.3 (1.2,1.4)	1.2 (1.1,1.3)	0.41
Adiponectin (μg/mL)[2]	10.4 (9.7,11.0)	9.8 (8.8,10.9)	0.74
TC (mmol/L)	5.6 (5.5,5.7)	5.8 (5.6,6.1)	0.09
TAG (mmol/L)[2]	1.3 (1.3,1.4)	1.4 (1.2,1.6)	0.64
HDL-C (mmol/L)[2]	1.4 (1.3,1.4)	1.3 (1.3,1.4)	0.81
Apo A1 (g/L)	1.2 (1.2,1.2)	1.2 (1.2,1.3)	0.68
LDL-C (mmol/L)	3.5 (3.4,3.6)	3.7 (3.5,3.9)	0.09
Apo B (g/L)	0.95 (0.29,0.02)	1.03 (0.33,0.04)	0.12

Values are mean (95% CI) or [2]GM (95% CI) at baseline. *P*-values for ANCOVA on total sample are shown, adjusted for age, gender and BMI. [1]*P*-values adjusted for gender and age.

4.3.7 *PPARG* Pro12Ala genotype associations with change in plasma lipid concentrations after dietary intervention

In order to investigate the effect of decrease in SFA without alteration in MUFA intake, we compared change in plasma lipid concentrations after continuation on the HS and switching to LF diet (18% *versus* 10% SFA), with respect to *PPARG* Pro12Ala genotype. Both diets contained the same proportion of PUFA. There was no significant difference in the change in either plasma TC, LDL-C or TAG concentration with respect to genotype (*n* =193), (*P* = 0.72, 0.60, 0.69 respectively after adjustment for change in BMI, age and gender as shown in **Table 4.7)**. To examine the effect of increased intake of MUFA without alteration in SFA, we compared change in plasma lipid concentrations after the HM and LF diets (20% *versus* 11% MUFA), which also contained the same proportion of PUFA. There was no significant difference in the change in either plasma TC, LDL-C or TAG concentration with respect to genotype (*n* =268), *P* = 0.74, 0.94, 0.43 respectively after adjustments as shown in **Table 4.7**.

Table 4.7 Change in plasma lipid concentrations after dietary interventions with respect to *PPARG* Pro12Ala

Phenotype		HS		LF		*P* diet x gene
		Pro/Pro (*n* = 47)	Pro/Ala + Ala/Ala (*n* =7)	Pro/Pro (*n* = 102)	Pro/Ala + Ala/Ala (*n* = 37)	
TC	**Baseline**	5.6 (5.3,5.8)	6.1 (5.4,6.8)	5.5 (5.3,5.7)	5.7 (5.4,6.1)	
	Follow-up	5.6 (5.3,5.9)	6.1 (5.3,6.9)	5.1 (5.0,5.3)	5.3 (5.0,5.6)	
	Change	-0.04 (-0.2,0.1)	-0.04 (-0.5,0.4)	-0.4 (-0.5,-0.3)	-0.5 (-0.7,-0.6)	0.72
TAG	**Baseline**	1.3 (1.2,1.5)	1.4 (1.1,1.9)	1.3 (1.2,1.4)	1.4 (1.2,1.6)	
	Follow-up	1.4 (1.2,1.6)	1.3 (1.0,1.8)	1.3 (1.2,1.4)	1.3 (1.1,1.5)	
	Change	0.04 (-0.04,0.11)	-0.07 (-0.31,0.16)	-0.01 (-0.06,0.04)	-0.06 (-0.16,0.05)	0.69
LDL-C	**Baseline**	3.5 (3.2,3.7)	4.0 (3.4,4.6)	3.5 (3.3,3.6)	3.7 (3.4,3.9)	
	Follow-up	3.5 (3.2,3.7)	4.1 (3.5,4.8)	3.2 (3.0,3.3)	3.4 (3.1,3.6)	
	Change	-0.03 (-0.2,0.10)	0.1 (-0.3,0.5)	-0.3 (-0.4,-0.2)	-0.3 (-0.4,-0.1)	0.6

Phenotype		HM		LF		*P* diet x gene
		Pro/Pro (*n* = 109)	Pro/Ala + Ala/Ala (*n* =20)	Pro/Pro (*n* = 102)	Pro/Ala + Ala/Ala (*n* = 37)	
TC	**Baseline**	5.7 (5.5,5.8)	5.9 (5.5,6.3)	5.5 (5.3,5.7)	5.7 (5.4,6.1)	
	Follow-up	5.3 (5.2,5.5)	5.5 (5.1,5.9)	5.1 (5.0,5.3)	5.3 (5.0,5.6)	
	Change	-0.3 (-0.4,-0.2)	-0.4 (-0.6,-0.1)	-0.4 (-0.5,-0.3)	-0.5 (-0.6,-0.3)	0.74
TAG	**Baseline**	1.3 (1.3,1.4)	1.4 (1.3,1.5)	1.3 (1.2,1.4)	1.4 (1.2,1.6)	
	Follow-up	1.4 (1.3,1.5)	1.4 (1.2,1.8)	1.3 (1.2,1.4)	1.3 (1.1,1.5)	
	Change	-0.01 (-0.06,0.04)	0.00 (-0.14,0.14)	-0.01 (-0.06,0.04)	-0.06 (-0.15,0.04)	0.43
LDL-C	**Baseline**	3.6 (3.5,3.7)	3.7 (3.4,4.0)	3.5 (3.3,3.6)	3.7 (3.4,3.9)	
	Follow-up	3.3 (3.2,3.4)	3.5 (3.1,3.8)	3.2 (3.0,3.3)	3.4 (3.1,3.6)	
	Change	-0.3 (-0.4,-0.2)	-0.3 (-0.6,-0.1)	-0.3 (-0.4,-0.2)	-0.3 (-0.4,-0.1)	0.94

Baseline, follow-up and change in mean plasma TC and LDL-C and mean GM TAG concentrations (95% CI) (mmol/L) after dietary interventions, stratified by *PPARG* Pro12Ala genotypes are shown. *P*-values adjusted for change in BMI, age and gender.

4.4 Discussion:

In this cohort of mainly overweight men and women, we have shown interaction between the habitual P:S ratio and *PPARG* Pro12Ala genotype influencing plasma TC and LDL-C and TAG concentrations. At low P:S ratio (≤0.33), mean TC and LDL-C concentrations in Ala12-allele carriers were significantly higher than in non-carriers. The trends for reduction in plasma TC and TAG concentrations with increasing P:S intake were significant in Ala12-allele carriers. Paired comparisons of outcomes after dietary intervention suggest that lower SFA intake was not responsible for the effect. A decrease in SFA intake after the HM and LF diets had no significant effect on either plasma TC, LDL-C or TAG concentrations.

Numerous studies have investigated associations between *PPARG* Pro12Ala and risk of obesity and diabetes, with equivocal outcomes. One meta-analysis of studies of T2D has found a significant increase in risk with the Pro12-allele (Altshuler et al, 2000) but another revealed no significant effect on related traits (Tonjes et al, 2006). Contrary findings of associations with obesity have also been reported (Masud & Ye 2003). These inconsistencies suggest that environmental modifiers of the effects of genetic variation in PPARγ2 may be involved. We have investigated associations between *PPARG* Pro12Ala genotype and concentrations of adiponectin, plasma lipid, obesity measures, fasting insulin and glucose and insulin sensitivity in subjects at risk of the metabolic syndrome, following habitual intake and dietary interventions differing in proportions of saturated and unsaturated fatty acids.

4.4.1 SNP associations at recruitment:

After the habitual diet, we found no significant differences in TC, LDL-C, HDL-C, TAG concentrations, fasting insulin, fasting glucose and HOMA-IR values. Pro/Pro homozygotes have higher BMI, waist circumference and percentage of body fat compared to Ala12-allele carriers **(Table 4.4)**. However the difference did not reach a level of significance ($P > 0.05$). Contrary findings of associations between obesity and Pro12Ala have also been reported. Our findings concerning obesity measures are in line with the original study by Deeb et al. (1998), where Ala12-allele carriers have a significantly lower BMI, and there is no difference in insulin sensitivity between genotypes. The lower transcriptional activity of the Ala variant *in vitro*, might account for reduced adipogenesis in Ala12-allele carriers, through the reduction in C/EBPα

expression (Lowell, 1999). However, other studies have failed to yield consistent findings, with some demonstrating a modest increase in BMI in Ala12-allele carriers (Beamer et al, 1998).

In White subjects, the dietary P:S ratio interacts with the genotype to influence concentrations of plasma TC and LDL-C and TAG (**Table 4.5** and **Figure 4.1)**. There is no significant interaction between *PPARG* Pro12Ala and dietary P:S ratio in determining HDL-C, fasting insulin, fasting glucose concentrations, HOMA-IR or measures of obesity. Memisoglu et al. (2003) found total fat intake was inversely correlated with plasma TC in Pro/Pro subjects but no effect among Ala12-allele carriers. They were the first to report an interaction between genotype and intake of MUFA, which was inversely associated with BMI in Ala12-allele carriers, but not in Pro/Pro homozygotes. Thus, the responsiveness of Ala12-carriers to dietary manipulation only emerged when MUFA rather than total fat intake was analysed. Luan et al. (2001) had previously shown greater sensitivity of Ala12-allele carriers to dietary PUFA in determination of BMI and fasting insulin. Without reference to diet, genotype was not significantly associated, but interaction between the P:S ratio and genotype in determining BMI or fasting insulin was highly significant (n = 592, P = 0.0038 and P = 0.0097). As the ratio of P:S increased, BMI and fasting insulin concentration decreased and in Ala12-allele carriers but not in Pro/Pro homozygotes. Both of these findings are compatible with unsaturated fats acting as specific ligands for PPARγ (Xu et al, 1999) and lower transcriptional activity of the PPARγ-Ala variant reducing PPARγ-mediated adipogenesis (Deeb et al, 1998).

Our study is the first to report significant interaction between the P:S ratio and *PPARG* Pro12Ala genotype influencing plasma TC and LDL-C and TAG concentrations. At low (<0.33) ratio of P:S in habitual intake, TC and LDL-C concentrations in carriers of the less transcriptionally active PPARγ-Ala variant were significantly higher than in those homozygous for the normal PPARγ-Pro form. As the P:S ratio increased, the concentration of plasma TC and LDL-C fell in Ala12-allele carriers, with a significant trend seen in the former. When PUFA replaces SFA in the diet, the major portion of cholesterol lowering is seen in the LDL fraction (Jebb et al, 2010). Increased plasma LDL-C has been observed following TZD treatment (Ovalle & Bell, 2002). The PPARγ-Ala12 form has lower transactivational ability than the wild-type (Deeb et al, 1998). However a mechanistic link to PPARγ target gene activation, that might infer

association of the less active PPARγ-Ala form with lower LDL-C concentration, has not been established.

Lipoprotein lipase activity is a rate-limiting determinant of TAG hydrolysis in plasma. Plasma TAG concentration in Ala12-allele carriers fell consistently beyond the second P:S quartile. It is well known that *n*-3 fatty acids decrease the concentration of serum TAG (Harris, 1997). PPARγ may mediate this effect, since PUFAs are PPARγ ligands (Xu et al, 1999) and *LPL* is a PPARγ target gene (Schoonjans et al, 1996). Lindi et al. (2003) found a significantly greater decrease in serum TAG concentration in healthy Ala12-allele carriers than in Pro/Pro homozygotes in response to *n*-3 fatty acid supplementation, when the total dietary fat intake was below 37% energy intake or the intake of SFA was below 10%. This is consistent with our finding of a fall in plasma TAG concentration in Ala12-allele carriers as P:S intake increased. However, both results suggest that the Ala12-allele is associated with increased LPL activity in the presence of high PUFA and by implication, higher transactivation of the *LPL* gene by the PPARγ Ala12- variant, contrary to findings *in vitro* (Deeb et al, 1998; Masugi et al, 2000). These functional studies suggest that the PPARγ Ala12-variant has a lower binding affinity to PPRE and consequently reduced transactivation ability. Lindi et al. (2003) found no difference in post-heparin plasma LPL activity following placebo or fatty acid supplementation, suggesting that any change in LPL expression elicited by PPARγ was not functionally significant. An explanation of the genotype x diet interaction which significantly influenced plasma TAG concentration is not evident at present.

We were not able to replicate Luan's findings in 2001, which reported a significant association between P:S ratio and *PPARG* Pro12Ala in determining BMI or fasting insulin. A meta-analysis in 2007 reported that the protective effects of the Ala12 allele in insulin resistance were stronger in individuals with a lower BMI (Ludovico et al, 2007); hence the higher BMI of the RISCK subjects (28.3 kg/m^2) may have attenuated this effect.

4.4.2 SNP associations at baseline:

The HS diet consumed during the 4-week run-in to baseline comprised 18% of energy SFA. The P:S intake ratio in White subjects at baseline was 0.35 i.e. in the second quartile of habitual P:S intake (0.34 – 0.47). At baseline, carriers of the Ala12-allele had higher concentrations of TC, LDL-C and Apo B and lower concentration of adiponectin compared to non-carriers, but differences were not significant after adjustment for BMI, gender and age. In the most recent meta-analysis including 52,998 subjects, only male Ala12-allele carriers had significantly increased plasma TC compared to non-carriers (Huang et al, 2011).

At recruitment and baseline, Ala12-allele carriers are more insulin sensitive when measured according to HOMA-IR, Si or HOMA2-IR, compared to Pro/Pro homozygotes. However, the differences did not reach significant ($P > 0.05$). These findings are in line with recent meta-analysis of findings, which report a protective effect of the Ala12-allele against T2DM (Huguenin & Rosa, 2010), but contradict others (Rhee et al, 2006). Carriers of Ala12-allele are reported to have higher serum adiponectin concentration (Yamamoto et al,2002; Mousavinasab et al,2005) and a lower β-cell function index, lower insulin secretion and higher levels of Haemoglobin A1C (Hba1c) (Mori et al, 2001). The later study evaluates the effect of *PPARG* Pro12Ala among a large number of Japanese individuals ($n = 3413$). Thus, the small sample size of our study is inadequate for detecting such a significant association

4.4.3 SNP associations after dietary intervention

In order to establish whether effects of interaction between with the P:S ratio of habitual intake and genotype that we had observed were related to increased PUFA, as distinct from decreased SFA, we first compared change in plasma lipid concentrations after HS and LF diets, in which SFA was reduced and MUFA remained constant. Carriage of the Ala12-allele was not significantly associated with change in either plasma TC, LDL-C or TAG concentrations, so the decrease in SFA had no significant effect. We obtained the same results when we compared changes in lipids after HM and LF diets, in which MUFA was raised and SFA remained constant. An increase in MUFA might have been expected to have had an effect, but they are weaker PPARγ activators than PUFAs (Xu et al, 1999). Therefore we cannot confirm that the interaction between the P:S ratio of habitual intake and *PPARG* Pro12Ala genotype in determining plasma TC, LDL-C, and

TAG concentrations depends specifically on an increase in consumption of PUFA, but it seems not to depend on a decrease in SFA.

Adiponectin gene expression is regulated by PPARγ and PUFAs which have been found to enhance the expression of adiponectin mRNA in adipose tissue and to dramatically increase plasma concentration of adiponectin (Maeda et al, 2001) (**Section 1.6.3)**. There was no significant difference in changes of adiponectin after HF, HM or LF diets with respect to *PPARG* Pro12Ala (**Appendix 5.2**). In comparison with PUFAs, MUFAs are weaker activators for PPARγ. Adiponectin concentrations at screening were not available. Therefore, we were not able to examine the interaction of P:S ratio of habitual intake and *PPARG* Pro12Ala as determinant of adiponectin concentration.

4.5 Limitations and conclusion

Limitations of our study include a relatively small number of genotyped subjects with plasma lipid measurements (n = 367) and the small observed changes in plasma lipid concentrations. To demonstrate a significant difference in LDL-C concentration in Ala12-allele carriers or non-carriers across all P:S quartiles, a total sample size of 1600 would be required for alpha = 0.05 and a power of 0.95. For TAG concentration, the equivalent sample size would be 1800. The significance of the effect of dietary P:S x gene interactions on plasma cholesterol and TAG concentrations should be treated with caution, as they were of modest significance in mainly overweight subjects. If substantiated in a larger cohort, a recommendation to Ala12-allele carriers to maintain a high dietary intake of PUFA: SFA, to reduce plasma concentrations of atherogenic cholesterol and TAG, would be justified. Identification of individuals who are genetically more likely to respond to particular dietary changes may be important for successful intervention in the prevention of CVD.

Chapter 5

The Effect of Interaction between *PPARG* Pro12Ala and *PPARA* Leu162Val and Dietary Intake of Fat on plasma lipids

5.1 Introduction

The peroxisome proliferator-activated receptors, members of the nuclear hormone receptor superfamily, are master transcriptional regulators of lipid and carbohydrate metabolism (Ferré, 2004). The major natural ligands of PPARγ and PPARα are PUFAs and prostanoids (Desvergne & Wahli 1999; Xu et al, 1999). PPARα responds to several fatty acids, suggesting roles in transducing nutritional into metabolic signals (Semple et al, 2006). PPARα plays an important role in liver, where it controls metabolism of lipoproteins and fatty acids, as well as glucose homeostasis (Lefebvre et al, 2006). Activation of PPARα by several fatty acids increases uptake and activates expression of genes involved in peroxisomal and mitochondrial fatty acid β-oxidation (Pyper et al, 2010), reducing the lipotoxic threat to insulin sensitivity. The PPARs are thus major regulators of lipid and glucose metabolism, allowing adaptation to nutritional status.

PPARA Leu162Val polymorphism involves a substitution of valine for leucine at codon 162 and has been associated with lipids and apolipoprotein concentrations (Vohl et al, 2000; Flavell et al, 2000). Other findings have suggested that the associations of the lipid profile with *PPARA* Leu162Val can be modulated by dietary fat intake (Robitaille et al, 2004). These inconsistencies suggest that environmental influences or gene-gene interaction may influence the outcome. Numerous studies have investigated associations between *PPARG* Pro12Ala and components of the metabolic syndrome alone or in interaction with dietary fat as previously explained in (**Section 4.1**). *In vitro* studies have shown that compared to the wild types, PPARγ-Ala12 form has lower binding affinity for PPREs (Deeb et al, 1998) and the PPARα-Val162 form has lower transcriptional activation in transfectants activated with ω-fatty acids (Rudkowska et al, 2009), thus both variants will reduce transactivational ability of the receptors.

We hypothesised that availability of the unsaturated fatty acid ligands of dietary origin might influence phenotypic outcomes based on carriage of the *PPARG* Pro12Ala and *PPARA* Leu162Val variants.

We aimed to test the following in RISCK study participants

1- Association between *PPARA* Leu162Val genotypes and mean quantitative phenotypes, including plasma lipids, adiponectin, fasting glucose and insulin, measures of obesity and insulin sensitivity at baseline. And to test this

association after a HS fat diet and after 24 weeks of dietary intervention in White subjects at risk of the metabolic syndrome.

2- Interaction between *PPARG* Pro12Ala and *PPARA* Leu162Val genotypes at baseline and their interaction with dietary fat in determining mean quantitative phenotypes at follow-up.

5.2 Subjects and methods

5.2.1 Subjects

This investigation was based on the participants of the RISCK study (**Section 2.1.1).**

5.2.2 Study design

Description of RISCK study design is given in **Section 2.1.1.**

5.2.3 Blood analytic methods and anthropometry measurement

For blood lipids, insulin sensitivity and anthropometric measurement are given in **Sections 2.3.1.2, 2.3.1.3 and 2.3.2** respectively.

5.2.4 DNA extraction and SNP genotyping

Details on methods for DNA extraction from buffy coats are given in **Section 2.3.4**. *PPARA* Leu162Val (rs1800206) was genotyped in the 466 participants for whom DNA was available and who had consented to genetic analysis using by Pyrosequencing. Primers and PCR conditions for genotyping are given in **Section 2.3.7**. Genotyping accuracy was 98% and success rate was 97%. The *PPARG* Pro12Ala SNP (rs1801282) was genotyped by KBiosciences (Hoddesdon, UK) (**Section 4.2.4)**.

5.2.5 Statistical analyses

All genotype distributions were tested for deviation from the Hardy-Weinberg equilibrium by a χ^2 test with 1 df ($P > 0.05$). Statistical analyses were carried out using the SPSS version 17.0 for Windows (SPSS Inc., Chicago, IL, USA). Values for Si, HOMA2-IR, adiponectin, insulin, TAG and HDL-C were log transformed to obtain better approximations of the normal distribution prior to analysis. Data were analysed using ANCOVA, regressing follow-up measures against baseline measures. Outliers were excluded from the ANCOVA and were defined as points > 2.5 times the interquartile range from the median on the transformed scale at baseline, follow-up, or change from baseline. All data presented in text and tables are expressed as means or GM ± SD or 95% CI. The effect of each diet is expressed as the follow-up value above the baseline with 95% CI. Statistical significance was taken at $P < 0.05$. ANCOVA was

used to test the interaction between genotypes and intervention diets. In this model, the dependant variable was the analysed follow-up plasma lipid; fixed factors were the combined genotypes and dietary intake of fat (HS, LF and HM). Baseline values, BMI, age and gender were the covariates. Figures were drawn using Prism version 5 (GraphPad software, Inc., California, and USA).

5.3 Results

5.3.1 Baseline characteristics of subjects

Section 3.3.1 and **Table 3.1** describe the characteristics of subjects who completed the study (*n* = 548). **Section 4.3.1** and **Table 4.1** describe the characteristics of subjects for whom DNA samples were available (*n* = 466).

5.3.2 Pyrosequencing template PCR optimisation

By comparing intensity of the bands produced by gel electrophoresis of the *PPARA* Leu162Val template PCR **Figure 5.1**, both optimal annealing temperature and $MgCl_2$ concentrations were determined. The expected length of the PCR product was 59 bp. Using the selected primers, an optimum PCR product was obtained with annealing temperature 54.0°C and 1.5mM $MgCl_2$ and 50 cycles (**Section 2.3.7**).

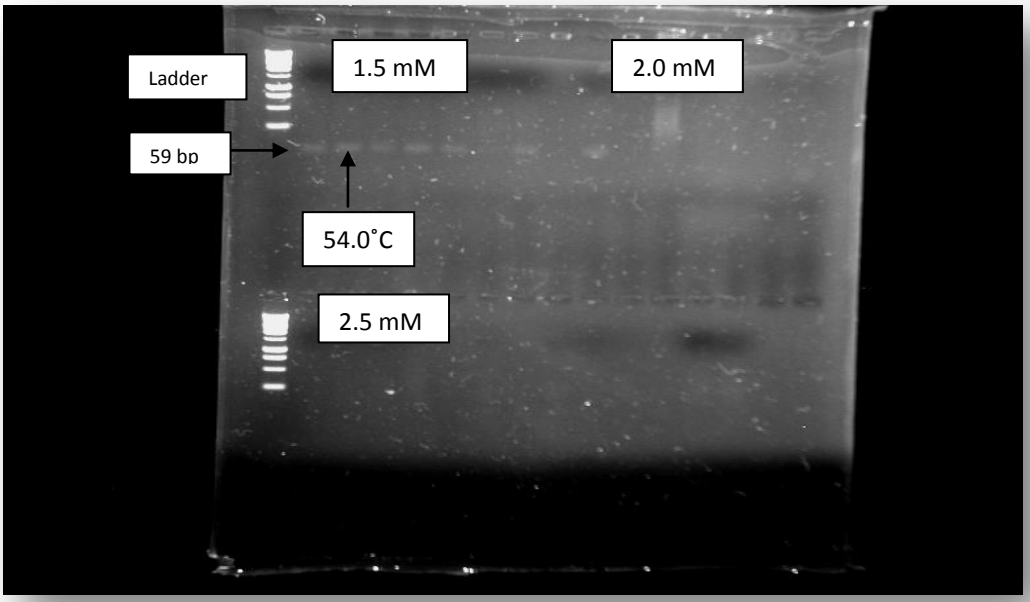

Figure 5.1 Gel electrophoresis of *PPARA* Leu162Val template PCR; bands of a specific length (59 bp) were produced at different anneal temperatures using 1.5mM, 2.0mM and 2.5mM $MgCl_2$, however strong bands were only produced at a concentration of 1.5 Mm $MgCl_2$. It appeared that the strongest band was produced at 54.0˚C.

5.3.3 Genotyping by Pyrosequencing

Pyrograms for the three *PPARA* Leu162Val genotypes are shown below (**Figure 5.2**). (For explanation of methodology see **Section 2.3.8)**

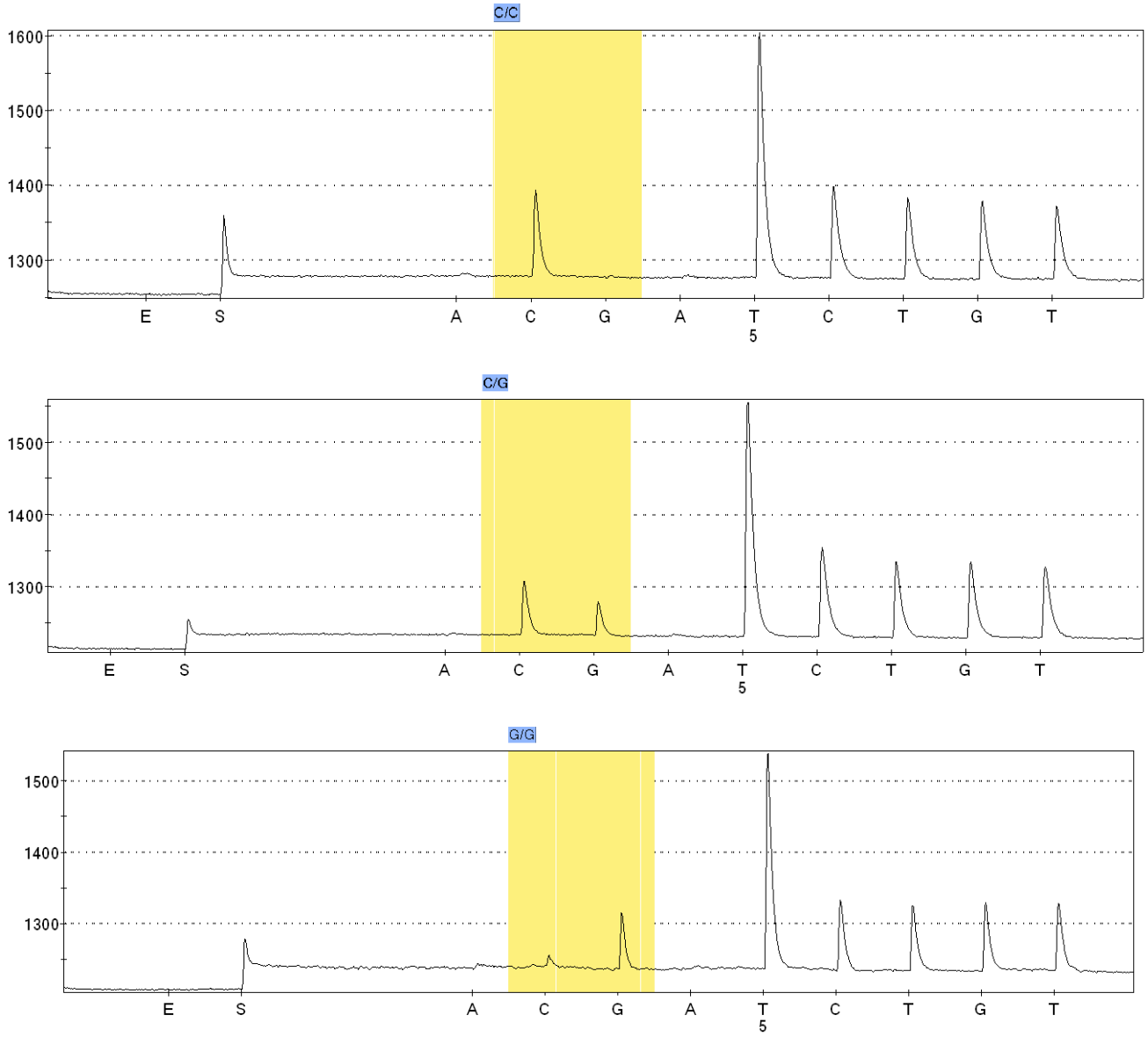

Figure 5.2 Pyrograms for Leu162Val genotypes Yellow blocks indicate the peaks for the analysed SNP.

5.3.4 SNP allele and genotype frequencies

Table 5.1 shows the allele and genotype frequencies for *PPARA* Leu162Val in the 466 RISCK subjects. Genotype distributions did not deviate from Hardy-Weinberg expectations. In comparison to MAFs listed on the NCBI SNP database (http://www.ncbi.nlm.nih.gov/snp build 132 accessed 01/09/11), the *PPARA* Val162 allele was also more frequent in RISCK White subjects (0.042 in HapMap-CEU) and absent in Blacks, as in HapMap-YRI trios. There is no comparative data available for the S. Asian subjects.

Table 5.1 *PPARA* Leu162Val allele and genotype frequency

	Ethnic group[1]				
	S. Asian [*n* (%)] 44 (9)	Black African [*n* (%)] 38 (8)	White European [*n* (%)] 367 (79)	Other [*n* (%)] 17(4)	All [*n* (%)] 466 (100)
MAF	0.05	0.0	0.06	0.03	0.06
Leu/Leu	39 (91)	37 (100)	310 (87)	16 (94)	402 (89%)
Leu/Val	4 (9)	0 (0)	46 (13)	1 (6)	51 (11%)
Val/Val	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Total	43 (9.5)	37 (8.2)	356 (78.6)	17 (3.7)	453 (100)

All subjects for whom DNA samples were available were genotyped (*n* = 466); *n* (%) is number of each ethnic group genotyped, as % of total. Total with genotype data (*n* = 453); *n* (%) refers to number of each genotype obtained, with % genotype frequency. [1]Self-reported ethnicity.

PPARG Pro12Ala allele and genotype frequency data in RISCK subjects were shown previously **(Section 4.3.2** and **Table 4.3)**. In view of the small sample size of the S. Asian and other ancestries and absence of the *PPARG* Pro12Ala and *PPARA* Leu162Val in Blacks, we chose to focus our genetic investigation on the White subjects only.

5.3.5 Independent associations of genotypes with concentrations of plasma lipids, adiponectin, fasting glucose and insulin, measures of obesity, and insulin sensitivity at baseline

Association between *PPARG* Pro12Ala genotypes and phenotypes can be found in **Section 4.3.6 and Table 4.6. Table 5.2** shows plasma lipids, adiponectin, fasting glucose and insulin, measures of obesity and insulin sensitivity at baseline, stratified *PPARA* Leu162Val genotypes. Carriers of Val126-allele have significantly higher BMI after adjusting for age and gender. They also have higher body fat % and waist circumference; however the differences did not achieve significant levels. There were no significant associations of *PPARA* Leu162Val genotype with plasma lipid concentrations, fasting glucose and insulin, Si or HOMA2-IR.

Table 5.2 Associations between *PPARA* Leu162Val genotypes and phenotypes at baseline in White subjects

Phenotype	*PPARA* Leu162Val genotype		
	Leu/Leu	Leu/Val	*P*
Males *n* (%)	131(42%)	20(43%)	
Females *n* (%)	179(58%)	26 (57%)	
BMI[1]	28.6 (28.1,29.1)	30.0 (28.6,31.4)	**0.05**
Waist circumference (cm)[1]	98.2 (96.8,99.6)	99.5 (95.8,103.1)	0.54
Body fat %[1]	33.7 (32.7,34.7)	34.6 (32.0,37.1)	0.49
Insulin sensitivity (IVGTT)((mU/L)-1 min-1)[2]	2.6 (2.4,2.9)	3.1 (2.2,3.8)	0.07
Fasting insulin (pmol/L)[2]	59.2 (55.5,63.0)	57.7 (49.0,68.0)	0.19
Fasting glucose (mmol/L)	5.7 (5.6,5.7)	5.9 (5.6,6.1)	0.29
HOMA2-IR[2]	1.3 (1.2,1.3)	1.2 (1.1,1.4)	0.24
Adiponectin (μg/mL) [2]	10.2 (9.6,10.7)	10.0 (8.5,11.9)	0.98
TC (mmol/L)	5.7 (5.6,5.8)	5.5 (5.2,5.8)	0.05
TAG (mmol/L)[2]	1.4 (1.3,1.5)	1.3 (1.2,1.5)	0.20
HDL-C(mmol/L)[2]	1.4 (1.3,1.4)	1.3 (1.3,1.4)	0.68
Apo A1 (mg/dL)	1.2 (1.2,1.3)	1.3 (1.2,1.3)	0.42
LDL-C (mmol/L)	3.6 (3.5,3.7)	3.5 (3.2,3.7)	0.09
Proportion of sdLDL (%)	24.9 (23.0,26.9)	24.4 (19.3,29.5)	0.58
Apol B (mg/dL)	1.0 (1.0,1.0)	1.0 (0.9,1.0)	0.43

Values are mean (95% CI) or [2]GM (95% CI) at baseline. *P*-values for ANCOVA on total sample are shown, adjusted for age, gender and BMI. [1]*P*-values adjusted for gender and age.

5.3.6 Effect of interaction between *PPARG* Pro12Ala and *PPARA* Leu162Val genotypes on concentrations of plasma lipids, adiponectin, fasting glucose and insulin, measures of obesity and insulin sensitivity at baseline in White subjects

Subjects for whom *PPARG* Pro12Ala and *PPARA* Leu162Val genotype data was available (n = 310) were divided into four genotype groups defined by presence or absence of the variant alleles. Mean plasma lipids, adiponectin concentrations, fasting glucose and insulin, insulin sensitivity and obesity measures with respect to genotype combinations are shown in **Table 5.3**. There was a significant interaction between genotypes as determinants of TC and TAG after adjustment for BMI, gender and age. Co-carriers of *PPARG* Ala12 and *PPARA* Val162 alleles were associated with higher plasma TC and TAG than in any other genotype group at baseline.

Table 5.3 Effect of interaction of *PPARA* Leu162Val and *PPARG* Pro12Ala on plasma lipid concentrations, obesity measures, fasting insulin and glucose, insulin sensitivity measures and in White subjects

	PPARA Leu162Val / *PPARG* Pro12Ala genotypes				*P*		
Phenotype	LL/PP (*n* = 215)	LL/PA+AA (*n* = 56)	LV /PP (*n* = 32)	LV /PA+AA (*n* = 7)	*PPARG* P12A	*PPARA* L162V	Interaction
BMI (kg/m^2)[1]	28.7 (28.1,29.4)	27.8 (26.6,29.1)	30.0 (28.4,31.6)	28.0 (24.5,31.5)	0.12	0.17	0.21
Waist circumference (cm)[1]	98.2 (96.6,99.9)	97.6 (94.3,100.9)	100.1 (95.7,104.5)	97.2 (88.0,106.4)	0.23	0.71	0.58
Body fat %[1]	34.2 (33.1,35.4)	31.6 (29.4,33.9)	34.3 (31.2,37.3)	30.1 (23.1,37.0)	0.17	0.62	0.57
Insulin sensitivity Si (IVGTT)((mU/L)-1 min-1)[2]	2.7 (2.5,2.9)	2.8 (2.4,3.3)	3.1 (2.5,3.8)	2.8 (1.8,4.3)	0.9	0.06	0.27
HOMA2-IR[2]	1.3 (1.2,1.4)	1.2(1.0,1.3)	1.2(1.0,1.4)	1.5 (1.0,2.2)	0.33	0.95	0.43
Adiponectin (μg/mL) [2]	10.3 (9.6,11.0)	10.2(9.1,11.3)	10.6(8.5,13.2)	7.4 (4.4,12.6)	0.74	0.55	0.58
Fasting insulin (pmol/L)[2]	4.1 (4.0,4.2)	4.0 (3.8,4.1)	3.9 (3.8,4.1)	4.4 (3.5,4.8)	0.63	0.17	0.13
Fasting glucose (mmol/L)	5.6 (5.5,5.7)	5.8 (5.6,6.0)	5.9 (5.6,6.2)	5.7 (5.1,6.3)	0.24	0.42	0.36
TC (mmol/L)	5.6 (5.5,5.8)	5.8 (5.6,6.1)	5.3 (4.9,5.6)	5.8 (5.1,6.5)	0.14	0.03	**0.04**
TAG (mmol/L)[2]	1.4 (1.3,1.5)	1.4 (1.2,1.5)	1.2 (1.0,1.4)	1.8 (1.3,2.5)	0.67	0.07	**0.03**
LDL-C(mmol/L)	3.6 (3.5,3.7)	3.7 (3.5,3.9)	3.3 (3.1,3.6)	3.7 (3.1,4.3)	0.13	0.08	0.12
Proportion of sdLDL (%)	24.6 (22.2,27.0)	25.6 (20.9,30.3)	23.9 (17.7,30.1)	34.4 (21.2,47.6)	0.74	0.78	0.7
Apo B (g/L)	0.97 (0.93,1.01)	1.02 (0.94,1.09)	0.87 (0.77,0.98)	1.08 (0.86,1.30)	0.18	0.14	0.15
HDL-C (mmol/L)[2]	1.4 (1.3,1.4)	1.4 (1.3,1.4)	1.3 (1.2,1.4)	1.2 (1.1,1.5)	0.83	0.71	0.95
Apo A1 (g/L)	1.2 (1.2,1.3)	1.2 (1.2,1.3)	1.2 (1.1,1.3)	1.3 (1.2,1.5)	0.74	0.46	0.54

Values are mean (95% CI) or [2]GM (95% CI) at baseline. All data and *P*-values for ANCOVA adjusted for age, gender and BMI, based on subjects for whom *PPARG* Pro12Ala and *PPARA* Leu162Val genotype data was available (*n* = 310). PP represents subjects homozygous for the *PPARG* Pro12 allele and PA + AA carriers of the Ala12 allele. LL represents subjects homozygous for the *PPARA* Leu162 allele and LV carriers of the Val162 allele.[1]P-values adjusted for gender and age.

5.3.7 Change in concentrations of measured variables after dietary intervention

Details on changes on plasma lipids and insulin sensitivity after dietary intervention can be found in **Section 3.3.7**.

5.3.8 Effect of interaction between genotypes on concentrations of plasma lipids after dietary intervention

Independent associations of *PPARG* Pro12Ala or *PPARA* Leu162Val genotypes with changes in concentrations of plasma lipids, adiponectin, fasting glucose and insulin, measures of obesity and insulin sensitivity measures with respect to baseline were not significant after randomisation to HM, LF or HS diets (**Appendix 5.1, 5.2, 5.3 and 5.4**).

Investigation of genotype interaction was based on subjects genotyped for both SNPs, with measured variables after HM (n =127) and LF (n =135) diets. There was a significant interaction between *PPARG* Pro12Ala and *PPARA* Leu162Val genotypes as determinants of plasma LDL-C, (P = 0.004) and sdLDL as a proportion of LDL (P = 0.012), after adjustment for change in BMI, age and gender. Co-carriers of the *PPARG* Ala12 and *PPARA* Val162 alleles had 47.2% lower LDL and 62.6% lower sdLDL as a proportion of LDL after the HM diet, compared to the LF diet. We did not test for an interaction after the parallel SFA diet, as there were no subjects carrying both *PPARG* Ala12 and *PPARA* Val162 alleles in this group. **Figure 5.2** shows the follow-up concentration of plasma LDL-C and sdLDL as a proportion of LDL-C after the HM and LF diets, adjusted for baseline concentrations, with respect to *PPARG* Pro12Ala and *PPARA* Leu162Val genotype combinations. We then analysed the data by excluding homozygous Ala/Ala subjects (n = 3), to ensure they did not skew the results when added to the heterozygote group. The interaction between diet and *PPARG* Pro12Ala and *PPARA* Leu162Val genotypes remained significant for LDL-C and sdLDL (P = 0.03 and 0.006 respectively).

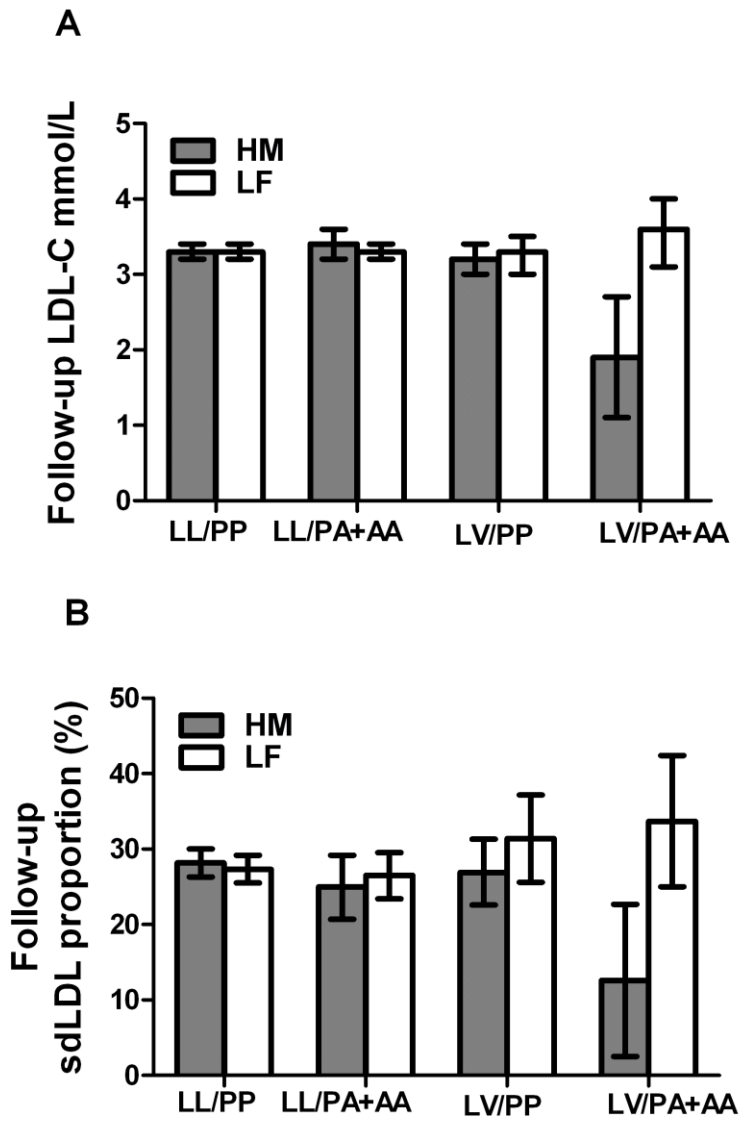

Figure 5.3 Interaction between *PPARG* Pro12Ala and *PPARA* Leu162Val genotype groups after dietary treatments influences plasma LDL-C concentration and sdLDL as proportion of LDL. Interaction between *PPARG* Pro12Ala and *PPARA* Leu162Val genotypes was a significant determinant of plasma concentrations of (A) LDL-C (P = 0.004) and (B) sdLDL as proportion of LDL (P = 0.012) after HM and LF diets, after adjustment for baseline values, change in BMI, age and gender using 3-way ANOVA. PP represents subjects homozygous for the *PPARG* Pro12 allele and PA + AA carriers of the Ala12 allele. LL represents subjects homozygous for the *PPARA* Leu162 allele and LV + VV carriers of the Val162 allele. Mean follow-up concentrations of LDL-C (mmol/L) and sdLDL (%) adjusted for baseline values after 24 weeks on HM or LF diets are shown. Bars indicate 95% CI. The figure is based on subjects with genotypes for both SNPs and measurements of plasma lipids after HM and LF diets. In HM diet, LL/PP n = 91, LL/LP+AA n =17, LV /PP n =16 and LV /PA+AA n =3. In LF diet LL/PP n = 89, LL/LP+AA n = 33, LV /PP n = 9 and LV /PA+AA n =4.

5.4 Discussion

Studies have reported that simultaneous activation of PPARα and PPARγ might alter the fatty acid distribution in liver, adipose tissues and skeletal muscle (Fiévet et al, 2006). PPARα activation will increase hepatic oxidation of fatty acids, reduce synthesis and secretion of TAG, decrease VLDL secretion, and lower VLDL delivery to peripheral tissues (Fiévet et al, 2006). Here we have shown independent and interactive effects of PPARγ2 gene *PPARG* Pro12Ala and PPARα gene *PPARA* Leu162Val genotypes in determination of plasma lipid concentrations, after diets lowering intake of SFA by replacement with MUFA or carbohydrate.

5.4.1 Independent associations of *PPARA* Leu162Val and *PPARG* ProAla12 genotypes with concentrations of plasma lipids, fasting glucose and insulin, measures of obesity and insulin sensitivity measures at baseline

After the 4 week HS diet, the *PPARA* Leu162Val genotype was not associated with adiponectin concentrations, insulin sensitivity or concentrations of plasma lipids at baseline. Others have reported similarly (Silbernagel et al, 2009; Doney et al, 2005), but associations of Val162 with higher (Sparsø et al, 2007) and lower (Nielsen et al, 2003) levels of plasma TAG have also been found. Others have also reported associations of Val162 with higher levels of Apo B and LDL-C (Vohl et al 2000; Tai et al, 2002) and HDL-C (Flavell et al, 2000). Some reports found a significant association of the Val162 with lower BMI (Evans et al, 2001). In our study Val162-allele carriers had significantly higher BMI compared to non-carriers. Animal studies showed that treatment with fibrate decreased body weight gain (Chaput et al, 2000). For *PPARG* Pro12Ala association at baseline refer to **Section 4.4.2**.

5.4.2 Interaction between genotypes on concentrations of plasma lipids, adiponectin, fasting glucose and insulin, measures of obesity and insulin sensitivity measures measurements at baseline

Co-carriers of *PPARG* Ala12 and *PPARA* Val162 alleles were associated with higher concentrations of plasma TC and TAG compared to other genotype group at baseline. One other investigation found no significant interaction between *PPARA* Leu162Val and *PPARG* Pro12Ala in determination of plasma lipid concentrations in obese subjects (Aberle et al, 2006). A shift from a mean ~13% of energy SFA in the habitual intake (Moore et al, 2009) to ~18% of energy SFA on the HS diet would be expected to

increase plasma cholesterol and TAG. As SFAs bind to PPARγ and PPARα with less affinity than PUFAs (Xu et al, 1999), increasing SFA would reduce activation of the receptors. *In vitro* studies have shown that compared to the wild types, PPARγ-Ala12 form has lower binding affinity for PPREs (Deeb et al, 1998) and the PPARα-Val162 form has lower transcriptional activation in transfectants activated with ω-fatty acids (Rudkowska et al, 2009), thus both variants will cause a reduce activation of the receptors.

5.4.3 Effect of interaction between genotypes on concentrations of plasma lipids, insulin sensitivity measures and obesity measures after dietary intervention

In mice, an increase in PPARγ mRNA in adipose tissue (Vidal-Puig et al, 1996), and increased hepatic PPARα mRNA and expression of PPARα target genes (Patsouris et al, 2006) after a high-fat diet, suggests that intake might influence expression of genes induced by both receptors in humans. Neither *PPARG* Pro12Ala nor *PPARA* Leu162Val genotypes were independently associated with changes in concentrations of plasma lipids, adiponectin concentration, obesity measures or insulin sensitivity measures after intervention. However, after randomisation, the power to detect significant changes was reduced. Various dietary interactions with Pro12Ala have been reported (Memisoglu et al, 2003; Luan et al, 2001); both findings are compatible with unsaturated fatty acids acting as specific ligands for PPARγ (Xu et al, 1999) and lower transcriptional activity of the PPARγ-Ala variant (Deeb et al, 1998) reducing adipogenesis. Reports of *PPARA* Leu162Val interaction with fatty acid intake in determination of plasma lipids are inconsistent, including no interaction with dietary fat (Robitialle et al, 2004), Val162 allele association with higher TC, LDL-C and Apo A1 after a high-PUFA diet (Paradis et al, 2005) and lower TAG and apoCII after high PUFA intake (Tai et al, 2005). Robitialle et al. (2004) reported a significant diet- gene interaction; with an increase in waist circumference in Leu162 homozygotes following high intake of dietary fat, no significant interaction was found in determining plasma lipids.

Our study is the first to report significant interaction between *PPARG* Pro12Ala and *PPARA* Leu162Val genotypes as a determinant of plasma concentrations of LDL-C concentration and proportion as sdLDL. After the HM diet there was a significantly larger reduction in plasma LDL-C concentration and proportion as sdLDL concentration in co-carriers of *PPARG* Ala12 and *PPARA* Val162 alleles compared to LF diet.

Fibrates and TZDs are agonists of PPARα and PPARγ respectively (Lefebvre et al, 2006; Belfort et al, 2006). In rats, insulin-induced gene *INSIG1*, the key regulator of SREBP activity, is up-regulated by activation of PPARγ in adipose tissue by rosiglitazone (Kast-Woelbern et al, 2004) and by activation of PPARα in liver by clofibrate (König et al, 2007). PPARγ activation by troglitazone has been shown to reduce nuclear SREBP-2 (Klopotek et al, 2006). mRNA concentration of the SREBP-2 target LDL receptor gene *LDLR* (Hua et al, 1993) is reduced after treatment with clofibrate (König et al, 2007). PPARγ activation by troglitazone has also been shown to down-regulate LDL clearance from plasma by the liver LDL receptor (Klopotek et al, 2006). If the same events were to be evoked by endogenous MUFA ligands in humans, activities of PPARγ and PPARα would tend to increase circulating LDL-C concentration.

Compared to wild-types, the PPARγ-Ala12 form has lower binding affinity for PPREs (Deeb et al, 1998) and the PPARα-Val162 form has lower transcriptional activation in transfectants activated with ω-fatty acids (Rudkowska et al, 2009). If both variants bound to the *INSIG1* PPRE with lower affinity, co-carriers of *PPARG* Ala12 and *PPARA* Val162 would express less Insig-1, SREBP-2 activity would be higher, and upregulation of the *LDLR* gene would lead to the maximum increase in LDL-C clearance from the plasma. There were no significant differences in concentrations between the other genotype combinations.

PPARG Ala12 carriers in the general Japanese population had a significantly higher proportion of sdLDL fractions 4-7, independent of lipid concentration (Hamada et al, 2007). We, however, found no independent association of Ala12 with sdLDL proportion, and the interaction with *PPARA* Val162 after the HM diet was associated with a reduction rather than an increase in sdLDL particles. Several studies have investigated the effects of different nutritional interventions on LDL particle size. Krauss & Dreon. (1995) investigated healthy men consuming high (46%) fat and low (24%) fat diets for 6 weeks in random order. After the high fat diet, 83% of subjects had predominantly large, buoyant LDL (>255 Å, the cut-off for sdLDL (Lamarche et al, 1999)), (pattern A), whereas the remainder had primarily smaller, denser LDL (pattern B). After the low fat diet, 41% with pattern A changed to B, i.e. the low fat diet increased the proportion of LDL as smaller, denser particles in some but not all

subjects, a variability that may reflect a genetic basis. In other studies, switching from a baseline diet rich in saturated fat to diets rich in MUFA, PUFA or a combination also reduced LDL particle size (Kratz et al, 2002). However, we found no significant change in the proportion of LDL-C as sdLDL after the switch from HS to HM or LF diets.

Bouchard-Mercier et al. (2011) found no significant change in LDL peak particle diameter (LDL-PPD) in *PPARG* Pro12 homozygotes or Ala12-allele carriers after high SFA intake, but a significant increase in LDL-PPD in Ala12-allele carriers after high intake of PUFA, which unlike SFA are PPARγ activators (Xu et al, 1999). We, however, found no independent association of Ala12 with sdLDL proportion either at baseline after run-in on HS diet, or after 24 weeks on HM or LF diets. Bouchard-Mercier et al. (2011) demonstrated dietary interaction with *PPARA* Leu162Val as a determinant of LDL-PPD. In agreement with reports of reduced LDL particle size on switching from SFA-rich to MUFA-rich diets (Kratz et al, 2002) they found in Leu162 homozygotes that LDL-PPD was larger after high SFA intakes than low. However, contrary to the overall trend, among Val162 carriers, subjects with higher SFA intakes had smaller LDL-PPD i.e. a higher proportion of sdLDL, than those with lower intakes. Fibrate ligands of PPARα can reduce production of VLDL (Shah et al, 2010) and lower sdLDL (Caslake et al, 1993), so in carriers of the less-active PPARα-Val form, activation by dietary ligands could result in a shift to a higher proportion of sdLDL. We found no significant reduction in the proportion of sdLDL in Val162 carriers compared to Leu162 homozygotes after dietary intervention, but a significant reduction in the proportion of sdLDL in carriers of both *PPARA* Val162 and *PPARG* Ala12 alleles after the HM diet. This cannot be explained by reduced activity of both variants, because as indicated above, this would be expected to lead to a higher proportion of sdLDL.

Only one other study has examined *PPARG* Pro12Ala and *PPARA* Leu162Val interaction after dietary intervention. After 2.5 years of low energy diet, in non-diabetic obese women there were significant favourable changes in lipid profile, but no significant interactive effects on anthropometric or biochemical characteristics at baseline or at the follow-up (Aldhoon et al, 2010).

5.5 Limitation and conclusion

Limitations include reduced power after randomisation to dietary treatments, compared to that at baseline, with small numbers carrying both variants (n =7). However, our ANOVA model used the variability of the whole dataset to measure the background variation and produced evidence of a significant effect of gene-gene interaction on LDL-C and proportion as sdLDL. The significance should nevertheless be treated with caution and confirmation awaits replication in a larger sample. Replication in another study with maximal correspondence in ethnic origin, age, and gender would be required to minimise the risk of false positive or negative gene-diet associations.

Chapter 6

The Effect of *PPARG* Pro12Ala and *PPARA* Leu162Val and n-3 LCPs Intake on Plasma Lipids

6.1 Introduction

Fish oil contains long-chain PUFA (LCP) which may act as physiological ligands for PPARγ and PPARα, suggesting roles in transducing nutritional into metabolic signals (Semple et al, 2006). Plasma TAG concentrations are lowered by increased intakes of n-3 LCP in a dose-dependent manner in the MARINA study (Sanders et al, 2011). Both EPA and DHA are known ligands for PPARα and PPARγ (Deckelbaum et al, 2006). Binding of n-3 fatty acids to PPARα and PPARγ induces β-oxidation and adipogenesis (Deckelbaum et al, 2006, Yu et al, 2011). *In vitro* studies have shown that both PPARγ-Ala12 and PPARα-Val162 variants are associated with reduced transactivational ability of the receptors (Deeb et al, 1998; Rudkowska et al, 2009).

The present study investigated the independent interaction of *PPARG* Pro12Ala and *PPARA* Leu162Val genotypes and dietary intake of n-3 PUFAs as determinants of plasma lipids in healthy participants of the MARINA study. It was hypothesised that *PPARG* Pro12Ala and *PPARA* Leu162Val genotypes interact with dietary intake of (n-3) LCPs EPA and DHA from fish, to influence concentrations of plasma lipids. We investigated the independent association between *PPARG* Pro12Ala and *PPARA* Leu162Val genotypes and mean quantitative phenotypes, including plasma lipids and BMI after a 4 week run-in on normal diet with placebo supplement, and after 12 months of dietary intervention in healthy subjects.

6.2 Subjects and Methods

6.2.1 Subjects

This investigation was based on participation in the MARINA study **(Section 2.1.2).**

6.2.2 Study design

Description of MARINA study design is given in **Section 2.1.2.**

6.2.3 Blood analytic methods:

Methods of blood lipid analysis are given in **Sections 2.3.1.2.**

6.2.4 DNA extraction and SNP genotyping

Details on methods for DNA extraction from buffy coats are given in **Section 2.3.4**. Genotyping by Pyrosequencing was performed on the 310 participants for whom DNA was available and who had consented to genetic analysis. Primers and PCR *PPARA* Leu162Val (rs1800206) genotyping are given in **Section 2.3.7**. Genotyping success rate was 93.8%. The *PPARG* Pro12Ala SNP (rs1801282) was genotyped by KBiosciences (Hoddesdon, UK) using the KASPar system. Genotype accuracy as assessed by inclusion of duplicates in the array was 98%, and negative controls (water blanks) were included on each plate. Genotyping success rate was 89%.

6.2.5 Statistical analyses

All genotype distributions were tested for deviation from the Hardy-Weinberg equilibrium by a χ^2 test with 1 df ($P > 0.05$). Statistical analyses were carried out using the SPSS version 17.0 for Windows (SPSS Inc., Chicago, IL, USA). TAG data were log transformed to obtain better approximations of the normal distribution prior to analysis. Data were analysed using ANCOVA, adjusting for baseline values as well as for age, gender and BMI. All data presented in the text and tables are expressed as means or GM ± SD or 95% CI. Statistical significance was taken at $P < 0.05$.

6.3 Results

6.3.1 Baseline characteristics of subjects

Out of 367 participants randomized to treatment, data was available for analysis on 312. The characteristics of all subjects who completed the study at baseline after a run-in on placebo supplement are shown in **Appendix 6.1**. The number of participants allocated to each of the four treatment groups did not significantly differ, nor did drop-out rates (Sanders et al, 2011). There were more women than men and most of the women were postmenopausal. About one-fifth of the study population was non-White with similar proportions of Asian and Black participants. The average BMI (25.6 kg/m^2) was above the desirable range (20-25 kg/m^2) and the mean waist circumference was greater than the cut-offs used to indicate risk of metabolic syndrome (94 cm in men and 80 cm in women) (Sanders et al, 2011). **Table 6.1** shows the characteristics of all subjects for whom DNA was available (*n* = 310) at baseline after a run-in on placebo supplement. There was no significant difference in all measured variables between the four treatment groups.

Table 6.1 Characteristics of all subjects at baseline by randomized treatment (*n* = 310)

	Placebo *n* = 70	0.45 g/day *n* = 80	0.9 g/day *n* = 80	1.8 g/day *n* = 80
Male	30 (42.8%)	30 (37.5%)	30 (37.5%)	29 (36.3%)
Female	40 (57.2%)	50 (62.5%)	50 (62.5%)	51 (63.7%)
Age (y)	55.4 (53.7,57.0)	55.1 (53.6,56.6)	55.2 (53.7,56.6)	55.1 (53.6,56.6)
BMI (kg/m^2)	26.1 (25.2,27.0)	25.2 (24.3,26.0)	26.2 (25.3,27.1)	25.1 (24.4,25.9)
TC:HDL-C[1]	3.6 (3.4,3.8)	3.6 (3.4,3.8)	3.4 (3.2,3.6)	3.4 (3.2,3.7)
TC (mmol/L)	5.4 (5.2,5.7)	5.4 (5.1,5.6)	5.4 (5.2,5.6)	5.4 (5.2,5.6)
TAG(mmol/L)[2]	1.14 (1.03,1.28)	1.14 (1.03,1.27)	1.13 (1.04,1.23)	1.12 (1.00,1.24)
HDL-C(mmol/L)	1.6 (1.5,1.7)	1.6 (1.5,1.6)	1.7 (1.5,1.8)	1.7 (1.6,1.8)
LDL-C(mmol/L)	3.3 (3.1,3.5)	3.2 (3.0,3.4)	3.2 (3.0,3.4)	3.2 (3.0,3.3)

Measurements made at baseline after a 4-week run-in on normal diet with placebo supplement and with (n-3) LCPs at the daily doses shown. Values are *n* (%) or mean or [2]GM (95% CI) and do not differ by treatment allocation.[1]TC:HDL-C molar ratio.

6.3.2 SNP minor allele and genotype frequencies

Table 6.2 shows the allele and genotype frequencies for *PPARG* Pro12Ala and *PPARA* Leu162Val in all subjects who completed the study, and for whom DNA was available (n = 310). Genotype distributions did not deviate from Hardy-Weinberg expectations. Minor allele frequencies in MARINA subjects were in close agreement with those listed for Europeans on the NCBI SNP database (Internet: http://www.ncbi.nlm.nih.gov/snp; build 132 accessed 15/04/11): HapMap-CEU: *PPARG* Ala12 0.13 and *PPARA* Val162 0.05.

Table 6.2 *PPARG* Pro12Ala and *PPARA* Leu162Val allele and genotype frequencies.

	Ethnic group[1]					
	Far East [*n* (%)][2] 9 (3)	S. Asian [*n* (%)] 18 (6)	Black African [*n* (%)] 16(5)	White European [*n* (%)] 254(82)	Other [*n* (%)] 13(4)	All [*n* (%)] 310(100)
PPARG Pro12Ala						
MAF	0.0556	0.25	0	0.1127	0.125	0.1141
Pro/Pro	8 (89)	9 (56)	13 (100)	167 (78)	9 (75)	206 (78)
Pro/Ala	1 (11)	6 (38)	0 (0)	44 (21)	3 (25)	54 (21)
Ala/Ala	0 (0)	1 (6)	0 (0)	2 (1)	0 (0)	3 (1)
Total	9 (100)	16 (100)	13 (100)	213 (100)	12 (100)	263 (100)
PPARA Leu162Val						
MAF	0	0	0	0.05	0	0.411
Leu/Leu	8 (100)	17 (100)	15 (100)	217 (90)	12 (100)	269 (92)
Leu/Val	0 (0)	0 (0)	0 (0)	22 (9)	0 (0)	22 (7)
Val/Val	0 (0)	0 (0)	0 (0)	1 (1)	0 (0)	1 (1)
Total	8 (100)	17 (100)	15 (100)	240 (100)	12 (100)	292 (100)

All subjects for whom DNA samples were available were genotyped (n = 310).[1]Self-reported ethnicity.[2]the number of each ethnic group genotyped, as % of total of number of each genotype obtained, with % genotype frequency.

In view of the absence of the *PPARA* Leu162Val in Blacks and Asians and *PPARG* Pro12Ala in Blacks we chose to focus our genetic investigation on the White subjects only. We also presented the data for all subjects.

6.3.3 Change in measured variables after dietary intervention

Erythrocyte fatty acid composition was determined to measure compliance to the dietary intervention. Capsule counts indicated 88.5% of the participants consumed more than 90% of the capsules provided. There were no statistically significant differences in the reported capsule intakes between treatment groups. The proportions of EPA and DHA in erythrocyte lipids increased in a dose-dependent manner, compared with placebo ($P < 0.001$ for trends), indicating long-term compliance to the intervention (Sanders et al, 2011).

No significant changes in the concentration of plasma TC or HDL-C were observed. However, plasma TAG concentrations were reduced by n-3 LCP consumption, compared to the placebo. The reduction in plasma TAG concentrations in all subjects and in males did not reach significance levels (Sanders et al, 2011). Among the female participants, there was a clear dose-response relationship ($n = 192$; $P = 0.002$ for trend) (Sanders et al, 2011). Plasma TAG concentrations were significantly lower following the highest dose (1.8 g/d) compared with the placebo and lower doses. The proportionate changes in females were -5.3% (95% CI -17.8, 7.3), -10.1% (95% CI-22.8, 2.5) and -24.0 % (95% CI -31.6, -11.3) for 0.45 g/d, 0.9 g/d and 1.8g/d (n-3) LCP respectively (Sanders et al, 2011).

6.3.4 Independent associations of genotypes with concentrations of plasma lipids and BMI at baseline

Table 6.3 and table 6.4 show plasma lipid measurements and BMI at baseline stratified by *PPARG* Pro12Ala and *PPARA* Leu162Val genotypes in all subjects and in White subjects only. After a 4-week run-in on normal diet with placebo supplement, there were no significant associations of *PPARG* Pro12Ala or *PPARA* Leu162Val genotypes with BMI or plasma lipid concentrations. These findings are in line with the RISCK findings, where no significant differences were found with lipid profile.

Table 6. 3 Associations between *PPARG* Pro12Ala and *PPARA* Leu162Val genotypes and phenotypes at baseline in all subjects

Phenotype	*PPARG* Pro12Ala			*PPARA* Leu162Val		
	Pro/Pro	Pro/Ala +Ala/Ala	*P*	Leu/leu	Leu/Val + Val/Val	*P*
Males *n*	75	28		107	8	
Females *n*	131	29		162	15	
BMI (kg/m^2)[1]	25.5 (25.0,26.0)	26.5 (25.6,27.5)	0.11	25.7 (25.2,26.2)	25.6 (23.9,27.4)	0.99
TC (mmol/L)	5.4 (5.2,5.5)	5.6 (5.3,5.9)	0.10	5.4 (5.3,5.5)	5.5 (5.1,5.9)	0.6
TAG (mmol/L)	1.1 (1.1,1.2)	1.2 (1.1,1.4)	0.43	1.1 (1.1,1.2)	1.1 (0.9,1.4)	0.75
HDL-C(mmol/L)	1.6 (1.6,1.7)	1.6 (1.5,1.7)	0.40	1.6 (1.6,1.7)	1.7 (1.5,1.9)	0.69
LDL-C (mmol/L)	3.2 (3.1,3.3)	3.4 (3.2,3.6)	0.18	3.2 (3.1,3.3)	3.3 (3.0,3.6)	0.69
TC:HDL	3.5 (3.4,3.6)	3.7 (3.5,3.9)	0.85	3.5 (3.4,3.6)	3.5 (3.1,3.8)	0.81

Values are mean (95% CI) [2]GM (95% CI) at baseline. *P*-values for ANCOVA on total sample are shown, adjusted for ethnicity, age, gender and BMI. [1]*P*-values adjusted for gender, ethnicity and age.

Table 6. 4 Associations between *PPARG* Pro12Ala and *PPARA* Leu162Val genotypes and phenotypes at baseline in White subjects

Phenotype	*PPARG* Pro12Ala			*PPARA* Leu162Val		
	Pro/Pro	Pro/Ala +Ala/Ala	*P*	Leu/leu	Leu/Val + Val/Val	*P*
Males *n*	61	22		88	8	
Females *n*	106	24		129	15	
BMI (kg/m^2)[1]	25.5 (24.9,26.1)	26.5 (25.4,27.5)	0.14	25.6 (25.1,26.1)	25.7 (23.9,27.4)	0.97
TC (mmol/L)	5.4 (5.3,5.6)	5.5 (5.2,5.8)	0.49	5.4 (5.3,5.5)	5.5 (5.1,6.0)	0.92
TAG (mmol/L)[2]	1.2 (1.1,1.2)	1.2 (1.0,1.4)	0.71	1.2 (1.1,1.2)	1.1 (0.9,1.4)	0.74
HDL- C (mmol/L)	1.7 (1.6,1.7)	1.6 (1.4,1.7)	0.76	1.6 (1.6,1.7)	1.7 (1.5,1.8)	0.78
LDL-C(mmol/L)	3.2 (3.1,3.3)	3.4 (3.1,3.6)	0.37	3.2 (3.1,3.3)	3.3 (2.9,3.7)	0.66
TC:HDL	3.5 (3.3,3.6)	3.7 (3.5,4.0)	0.43	3.5 (3.4,3.6)	3.5 (3.1,3.8)	0.92

Values are mean (95% CI) [2]GM (95% CI) at baseline. *P*-values for ANCOVA on total sample are shown, adjusted for age, gender and BMI. [1]*P*-values adjusted for gender and age.

6.3.5 Associations of *PPARG* Pro12Ala and *PPARA* Leu162Val genotypes with changes in plasma lipid concentrations after dietary intervention in White subjects

Plasma lipid concentrations were measured in the four treatment groups (placebo and three n-3 LCP doses) at baseline and at 6 and 12 months after treatment. The average of the measurements at 6 and 12 months was then subtracted from those made at baseline to determine the average change. The treatment effect was then determined with respect to the average measurement obtained after the placebo, i.e. the difference between the average measure after each dose and the average after the placebo. Interaction between treatment and *PPARG* Pro12Ala was not significant in determining any of the blood lipid variables, including TC, HDL-C or LDL-C (P>0.05) after adjustment for all and White subjects as shown in **Appendices 6.2 and 6.3**. No significant interaction between treatment and *PPARA* Leu162Val determined any of the blood lipid variables including TC, TAG, HDL-C or LDL-C (P>0.05) after adjustment for all and White subjects as shown in **Appendices 6.4 and 6.5.**

Interaction between *PPARA* Leu162Val and treatment was a significant determinant of plasma TAG concentrations (P = 0.04) after adjusting for age, BMI and gender for White subjects and (P = 0.043) for all subjects after adjusting for age, BMI, gender and ethnicity. As shown in **Tables 6.5 and 6.6**, treatment lowered plasma TAG concentration significantly in Leu162 homozygotes, (P =0.001) but not in Val162-allele carriers (P =0.27). Increasing the dose of n-3 LCP (0.45 g/d to 1.8 g/d) and increasing the period of treatment (6 months to 12 months) caused a progressive decrease in plasma TAG concentrations. The same effect was seen in White subjects. No significant interaction between *PPARG* Pro12Ala and treatment was found in determining plasma TAG concentrations as shown in **Tables 6.7 and 6.8**. We then analysed the data by excluding homozygous Ala/Ala subjects (n = 2) and homozygous Val/Val subjects (n = 1), to ensure they did not skew the results when added to the heterozygote group. The interaction between treatment and *PPARA* Leu162Val genotypes remained significant for TAG in all and White subjects only (P = 0.041 and 0.046 respectively), while the interaction with *PPARG* Pro12ala remained insignificant for all and White subjects only (P>0.05).

We were also interested to replicate the findings of the interaction between *PPARG* Pro12Ala and *PPARA* Leu162Val in the RISCK study. However, in the MARINA study only one subject proved to be the carrier of the minor variants of both SNPs, thus preventing us from looking at their interaction.

Table 6.5 Plasma TAG concentrations stratified by *PPARA* Leu162Val genotype after n-3 LCP treatment in all subjects

PPARA Leu162Val	TAG (mmol/L)	Placebo	0.45g/day	0.9g/day	1.8g/day	*P*
Leu162 homozygotes		*n* = 61	*n* = 73	*n* = 66	*n* = 69	
	Baseline	1.13 (1.00,1.27)	1.12 (1.00,1.24)	1.14 (1.04,1.25)	1.15 (1.03,1.28)	
	6 months	1.19 (1.08,1.31)	1.10 (0.97,1.24)	1.08 (0.98,1.20)	0.95 (0.86,1.06)	
	12 months	1.20 (1.08,1.34)	1.14 (1.04,1.25)	1.15 (1.05,1.25)	0.97 (0.87,1.08)	
	Average	1.21 (1.10,1.33)	1.14 (1.04,1.25)	1.12 (1.03,1.23)	0.97 (0.88,1.07)	
	Change	0.07 (-0.05,0.10)	0.02 (-0.10,0.07)	-0.02 (-0.23,-0.07)	-0.15 (-0.06,0.02)	
	Treatment effect	0.000 [Reference]	-0.054 (-0.184,0.077)	-0.071 (-0.205,0.063)	-0.217 (-0.350,-0.084) *	<0.001
Val162 carriers		*n* = 6	*n* = 4	*n* = 7	*n* = 6	
	Baseline	1.31 (1.00,1.73)	1.43 (0.68,3.01)	1.28 (0.80,2.06)	0.74 (0.41,1.32)	
	6 months	0.98 (0.61,1.57)	1.58 (0.52,4.79)	1.39 (0.88,2.20)	0.84 (0.52,1.36)	
	12 months	1.18 (0.89,1.57)	1.58 (0.67,3.71)	1.44 (0.89,2.34)	0.70 (0.56,0.88)	
	Average	1.13 (0.85,1.49)	1.59 (0.60,4.23)	1.44 (0.95,2.21)	0.78 (0.55,1.11)	
	Change	-0.15 (-0.43,0.13)	0.11 (-0.18,0.39)	0.12 (-0.02,0.26)	0.06 (-0.22,0.33)	
	Treatment effect	0.000 [Reference]	0.260 (-0.04,0.56)	0.273 (0.01,0.53)	0.208 (-0.06,0.48)	0.27

Table 6.6 Plasma TAG concentrations stratified by *PPARA* Leu162Val genotype after n-3 LCP treatment in White subjects

PPARA Leu162Val	TAG (mmol/L)	Placebo	0.45g/day	0.9g/day	1.8g/day	*P*
Leu162 homozygotes		*n* = 49	*n* = 57	*n* = 52	*n* = 59	
	Baseline	1.17 (1.02,1.35)	1.09 (0.97,1.23)	1.19 (1.07,1.32)	1.18 (1.05,1.33)	
	6 months	1.21 (1.09,1.35)	1.12 (0.97,1.29)	1.09 (0.97,1.23)	0.96 (0.86,1.08)	
	12 months	1.21 (1.07,1.37)	1.17 (1.06,1.30)	1.17 (1.06,1.30)	0.99 (0.88,1.12)	
	Average	1.23 (1.10,1.36)	1.17 (1.06,1.30)	1.14 (1.03,1.26)	0.98 (0.88,1.10)	
	Change	0.05 (-0.06,0.15)	0.07 (-0.01,0.15)	-0.04 (-0.14,0.06)	-0.17 (-0.25,-0.08)	
	Treatment effect	0.000 [Reference]	0.023 (-0.11,0.15)	-0.085 (-0.22,0.05)	-0.212 (-0.34,-0.08)*	0.001
Val162 carriers		*n* = 6	*n* = 4	*n* = 7	*n* = 6	
	Baseline	1.31 (1.00,1.73)	1.43 (0.68,3.01)	1.28 (0.80,2.06)	0.74 (0.41,1.32)	
	6 months	0.98 (0.61,1.57)	1.58 (0.52,4.79)	1.39 (0.88,2.20)	0.84 (0.52,1.36)	
	12 months	1.18 (0.89,1.57)	1.58 (0.67,3.71)	1.44 (0.89,2.34)	0.70 (0.56,0.88)	
	Average	1.13 (0.85,1.49)	1.59 (0.60,4.23)	1.44 (0.95,2.21)	0.78 (0.55,1.11)	
	Change	-0.15 (-0.43,0.13)	0.11 (-0.18,0.39)	0.12 (-0.02,0.26)	0.06 (-0.22,0.33)	
	Treatment effect	0.000 [Reference]	0.260 (-0.04,0.56)	0.273 (0.01,0.53)	0.208 (-0.06,0.48)	0.27

For tables 6.5 and 6.6 Log plasma TAG concentrations (95% CI) (mmol/L) are shown. Data is presented for *PPARA* Leu162 homozygous and *PPARA* Val162-allele carrier in all and White subjects, for whom DNA samples and plasma TAG measurements were available. All variables were measured at baseline after a 4-week run-in on normal diet with a placebo supplement and after 6 and 12 months, during which normal diet was supplemented with n-3 LCP at the daily doses shown. The average of the measurements at 6 and 12 months was subtracted from those made at baseline to determine the average change. Treatment effects were determined by univariate ANCOVA. *P*-values derived from the GM values are presented. *P*-values are adjusted for BMI, age, ethnicity and gender for all subjects and *P*-values are adjusted for BMI, age and gender for White subjects (nominally significant at $P < 0.05$).* significant difference between the highest dose of n-3 PUFA and placebo. Treatment x *PPARA* Leu162Val *P*=0.043 for all subjects and *P*=0.04 for White subjects.

Table 6.7 Plasma TAG concentrations stratified by *PPARG* Pro12Ala genotype after n-3 LCP treatment in all subjects

PPARG Pro12Ala	TAG (mmol/L)	Placebo	0.45g/day	0.9g/day	1.8g/day	*P*
Pro12 homozygotes		*n* = 46	*n* = 49	*n* = 54	*n* = 57	
	Baseline	1.11 (0.98,1.26)	1.16 (1.01,1.33)	1.12 (1.01,1.24)	1.13 (0.99,1.29)	
	6 months	1.19 (1.05,1.34)	1.10 (0.94,1.29)	1.05 (0.94,1.18)	0.95 (0.84,1.07)	
	12 months	1.17 (1.03,1.32)	1.16 (1.03,1.30)	1.17 (1.05,1.31)	0.96 (0.85,1.08)	
	Average	1.19 (1.06,1.33)	1.16 (1.03,1.30)	1.12 (1.01,1.25)	0.96 (0.86,1.07)	
	Change	0.07 (-0.09,0.08)	0.00 (-0.10,0.10)	0.00 (-0.24,-0.05)	-0.15 (-0.07,0.02)	
	Treatment effect	0.000 [Reference]	-0.028 (-0.191,0.134)	-0.058 (-0.217,0.100)	-0.214 (-0.372,-0.057)	0.025
Ala12 carriers		*n* =13	*n* = 19	*n* = 9	*n* =16	
	Baseline	1.52 (1.14,2.02)	1.22 (0.94,1.57)	1.10 (0.87,1.38)	1.07 (0.83,1.38)	
	6 months	1.26 (1.02,1.55)	1.27 (0.98,1.63)	1.31 (1.00,1.73)	0.96 (0.76,1.22)	
	12 months	1.36 (1.12,1.65)	1.23 (0.98,1.53)	1.14 (0.80,1.63)	0.92 (0.71,1.20)	
	Average	1.32 (1.10,1.58)	1.26 (1.01,1.57)	1.23 (0.92,1.63)	0.96 (0.76,1.21)	
	Change	-0.14 (-0.14,0.20)	0.03 (-0.16,0.38)	0.11 (-0.25,0.03)	-0.11 (-0.12,0.06)	
	Treatment effect	0.000 [Reference]	-0.045 (-0.338,0.247)	-0.074 (-0.427,0.279)	-0.319 (-0.623,-0.015)	0.19

Table 6. 8 Plasma TAG concentrations stratified by *PPARG* Pro12Ala genotype after n-3 LCP treatment in White subjects

PPARG Pro12Ala	TAG (mmol/L)	Placebo	0.45g/day	0.9g/day	1.8g/day	*P*
Pro12 homozygotes		*n* = 38	*n* = 39	*n* = 43	*n* = 47	
	Baseline	1.17 (1.01,1.34)	1.18 (1.01,1.37)	1.17 (1.03,1.32)	1.15 (0.99,1.32)	
	6 months	1.22 (1.06,1.40)	1.13 (0.93,1.37)	1.06 (0.93,1.22)	0.93 (0.81,1.06)	
	12 months	1.19 (1.04,1.36)	1.19 (1.05,1.35)	1.19 (1.05,1.35)	0.95 (0.83,1.08)	
	Average	1.22 (1.08,1.38)	1.19 (1.05,1.36)	1.14 (1.01,1.28)	0.94 (0.83,1.07)	
	Change	0.05 (-0.08,0.11)	0.02 (-0.14,0.09)	-0.02 (-0.27,-0.07)	-0.17 (-0.09,0.01)	
	Treatment effect	0.000 [Reference]	-0.022 (-0.201,0.157)	-0.070 (-0.245,0.104)	-0.256 (-0.428,-0.084)	0.03
Ala12 carriers		*n* =10	*n* = 14	*n* = 6	*n* =15	
	Baseline	1.55 (1.07,2.23)	1.18 (0.85,1.62)	1.10 (0.79,1.51)	1.09 (0.83,1.43)	
	6 months	1.23 (0.95,1.58)	1.31 (0.95,1.79)	1.37 (0.92,2.04)	0.98 (0.76,1.27)	
	12 months	1.36 (1.08,1.72)	1.29 (0.98,1.71)	1.27 (0.84,1.93)	0.95 (0.73,1.24)	
	Average	1.31 (1.05,1.63)	1.31 (0.99,1.74)	1.31 (0.91,1.89)	0.98 (0.78,1.25)	
	Change	-0.17 (-0.09,0.31)	0.11 (-0.17,0.52)	0.18 (-0.25,0.04)	-0.10 (-0.12,0.10)	
	Treatment effect	0.000 [Reference]	0.003 (-0.350,0.356)	0.001 (-0.419,0.421)	-0.284 (-0.632,0.064)	0.41

For tables 6.7 and 6.8 Log plasma TAG concentrations (95% CI) (mmol/L) are shown. Data is presented for *PPARG* Pro12 homozygous and *PPARG* Ala12-allele carrier in all and White subjects, for whom DNA samples and plasma TAG measurements were available. All variables were measured at baseline after a 4-week run-in on normal diet with a placebo supplement and after 6 and 12 months, during which normal diet was supplemented with n-3 LCP at the daily doses shown. The average of the measurements at 6 and 12 months was subtracted from those made at baseline to determine the average change. Treatment effects were determined by univariate ANCOVA. *P*-values are adjusted for BMI, age, ethnicity and gender for all subjects and *P*-values are adjusted for BMI, age and gender for White subjects (nominally significant at $P < 0.05$) (nominally significant at $P < 0.05$). Treatment x *PPARG* Pro12Ala $P=0.87$ for all subjects and $P=0.6$ for White subjects

6.4 Discussion

The MARINA study was designed to test the effects of three dose levels of n-3 long-chain polyunsaturated fatty acids: n-3 LCPs, EPA and DHA versus placebo on variables associated with the risk of cardiovascular disease. Treatment caused a significant reduction in plasma TAG concentration in females, but not in males (Sanders et al, 2011). We found a treatment effect related to genotype for SNP in the gene for PPARα. Plasma TAG concentration decreased in all subjects homozygous for the *PPARA* Leu162 alleles. A significant interaction between n-3 LCP treatment and genotypes of *PPARA* Leu162Val SNPs influenced plasma TAG concentration.

The effects of fish oils rich in n-3 fatty acids in lowering TAGs are well known (Harris, 1997). The n-3 LCPs EPA and DHA have a relatively potent effect on plasma lipids compared with other fatty acids, with effects noted with doses as low as 0.7 g/d (Theobald et al, 2007). After treatment, TAG concentration was significantly reduced in all MARINA subjects and particularly so in females, but did not reach significance in males. The highest dose lowered plasma TAG concentrations in women by 24%, with clear evidence of a dose-response relationship. The reduced effect in men may reflect the smaller number of participants. However, as we found no significant treatment x *PPARA* Leu162Val or *PPARG* ProAla12 x gender we did not test association in males and females separately.

6.4.1 Independent associations of *PPARA* Leu162Val and *PPARG* ProAla12 genotypes with concentrations of plasma lipids and obesity measures at baseline

After a 4 week run-in on a normal diet with a placebo supplement, the *PPARA* Leu162Val genotype did not prove to be associated with concentrations of plasma lipids or BMI at baseline. Others have reported similar results to these (Silbernagel et al, 2009; Doney et al, 2005). *PPARG* Pro12Ala was also not associated with lipid concentrations. Neither were we able to find an association with BMI. There is an insignificant trend for BMI to be higher in Ala12-allele carriers, which support the findings of some studies (Beamer et al, 1998) and contradict others (Deeb et al, 1998).

6.4.2 Independent associations of *PPARA* Leu162Val and *PPARG* ProAla12 genotypes with concentrations of plasma lipids after dietary intervention

We found no significant interaction between plasma lipid concentration and *PPARG* Pro12Ala genotypes, after 12 months of n-3 supplementation, in determining lipid profile. This was similar to our observation in the RISCK study, in which 6 months of high MUFA intake did not significantly interact with *PPARG* Pro12Ala to determine concentration of any of the plasma lipids **(Section 5.3.8)**. We have reported a significant interaction between habitual P:S ratio and *PPARG* Pro12Ala genotype-influencing plasma LDL-C, TC and TAG concentrations. Recently, Lamri et al. (2011) reported a significant interaction between *PPARG* Pro12Ala and habitual intake of dietary fat in determining BMI. In this study, Ala12 homozygotes had significantly higher BMI than Pro-allele carriers among the high fat consumers. Their results are in line with Luan's findings, where low habitual P:S ratio is associated with higher BMI in Ala12-allele carriers but nor Pro homozygotes (Luan et al, 2001). It seems that relatively long term exposure to PUFAs i.e. habitual intake is required in order to observe any change in plasma lipid concentrations or BMI with respect to *PPARG* Pro12Ala genotype. As reported previously (**Section 4.1**), the PPARγ2 Ala-variant exhibits modest impairment of transcriptional activation following treatment with pharmacological ligand TZDs (Deeb et al, 1998; Masugi et al, 2000). Ala12–allele carriers may be more responsive to long term intake of PUFAs rather than short term dietary manipulations. Low doses of n-3 LCPs (0.45, 0.9 or 1.8 g/d) used in the MARINA study may also affect the non-significant interaction with *PPARG* Pro12Ala. Higher doses of 3.6 g n-3 fatty acids/day significantly decrease serum TAG concentration in healthy Ala12-allele carriers compared to Pro/Pro homozygotes (Lindi et al, 2003). The lack of effect of *PPARG* Pro12Ala is consistent with the fatty acids being weak ligands for PPARγ compared to PPARα (Xu et al, 1999).

Reports of *PPARA* Leu162Val interaction with fatty acid intake in determining plasma lipids are inconsistent, including no interaction with PUFA (Tai et al, 2005), Val162-allele association with higher total and LDL-C after a high-PUFA diet (Paradis et al, 2005), and higher TAG after low PUFA intake (Tai et al, 2005). We found that *PPARA* Leu162Val genotypes were not associated with changes in concentrations of plasma lipids after intervention in the RISCK study (**Section 5.3.5**). However, as PUFAs are stronger ligands of PPARα than MUFAs (Xu et al, 1999), this may account for

significant interaction between n-3 LCP treatment and genotypes of *PPARA* Leu162Val influencing plasma TAG concentration. Treatment lowered plasma TAG concentration significantly in Leu162 homozygotes, with significant increase between the highest dose compared to placebo but not in Val162-allele carriers.

Most studies have found a reduced production rate of VLDL with n-3 LCP consumption in humans (Harris & Bulchandani, 2006), so part of the mechanism is likely to involve the inhibition of hepatic VLDL secretion. VLDL TAG synthesis can be reduced by decreasing fatty acid availability, secondary to an increase in β-oxidation, or by a decrease in lipogenesis (i.e. fatty acid synthesis). PPARα activation would suppress hormone sensitive lipase and decrease NEFA concentrations (Jia et al, 2011). An alternative mechanism through which n-3 LCPs could influence TAG concentration could be the potential enhancement of clearance by lipoprotein lipase. Park & Harris, (2003) have found EPA and DHA were equally effective in reducing postprandial TAG concentration and chylomicron triglyceride half-lives, while chylomicron particle sizes and lipoprotein lipase activity was increased. These results suggest that n-3 LCP supplementation accelerates chylomicron TAG clearance by increasing LPL activity. The *LPL* gene is responsive to PPARα activation (Schoonjans et al, 1996). Furthermore, after n-3 fatty acid dietary supplementation, Rudkowska et al, (2010) found LPL activity increased more in *PPARA* Leu162 homozygotes than in Val162-allele carriers and LPL activities were inversely related to plasma TAG concentration. Transcription was also found to be higher in Leu162- than Val162-constructs containing the *LPL* PPRE, after *n*-3 fatty acid transactivation (Rudkowska et al, 2009). These findings are compatible with the decreased TAG concentration we found in subjects homozygous for the more transcriptionally active Leu162 allele.

6.5 Limitation and conclusion

Limitations of our study include a relatively small number of genotyped subjects with plasma lipid measurements (n = 310) and the small observed changes in plasma lipid concentrations and relatively low doses of n- LCP used. Significant interactions between n-3 LCP treatment and genotypes of *PPARA* Leu162Val SNP were found to contribute to a reduction in plasma TAG concentration. The modulating effects of n-3 LCP on plasma lipids were evident at doses of 0.45-1.8 g/d in the MARINA subjects. The discovery of significantly different responses to treatment in genetic subgroups

found in the current study has potential application in progress toward a personalized approach to prevention of CVD.

Chapter 7

The Effect of Supplementation with EPA or DHA on Serum Adiponectin Concentrations

7.1 Introduction

Numerous studies have investigated the effect of fish oils, especially DHA and EPA on risk factors for cardiovascular disease (Harris et al, 2008). However, fewer studies have investigated their differential effect of EPA *versus*. DHA. Animal studies suggest that both EPA and DHA taken individually exert an insulin-sensitising action (Murata et al, 2001; Shimura et al, 1997), but the results from human feeding studies have been equivocal (Woodman et al, 2002; Griffin et al, 2006; Vessby et al, 2001; Egert et al, 2008). A recent report from the LIPGENE investigators, suggested a beneficial effect of long chain n-3 supplementation on features metabolic syndrome (Paniagua et al, 2011). Some of the variability may be due to the differing proportions of EPA and DHA used in the studies.

In mice, an improvement in insulin sensitivity and an increase in adiponectin concentrations with DHA but not EPA was noted (Vemuri et al, 2007). Furthermore, in 3T3-L1 adipocytes, DHA, but not EPA, increases adiponectin concentration through a PPARγ pathway (Oster et al, 2010; Tishinsky et al, 2011). The *in vitro* and animal experimental data suggest that DHA will increase serum adiponectin concentrations. Long chain *n-3* PUFA are known ligands for PPAR-γ (Maeda et al, 2001), while adiponectin gene expression is modulated by PPAR-γ (Iwaki et al, 2003). Fish oil was reported to increase adiponectin concentrations in human (Krebs et al, 2006) and animal studies (Hassanali et al, 2010; Kuda et al 2009; Duda et al, 2009; Higuchi et al, 2008). In PPARγ transgenic mice, fish oil rich in EPA and DHA markedly increased plasma adiponectin concentrations (Yu et al, 2011). However, a study in diabetic mice revealed that supplementation of EPA (1 g/kg body weight per day) or fish oil for 11 months had no effect on fasting adiponectin concentrations, fasting insulin or glucose (Cummings et al, 2010).

In vitro, EPA exposure up-regulates the IRS-1 associated with the activation of PI3K and Akt kinase and down-regulates gluconeogenesis (Murata et al, 2001). In KK-Ay mice, DHA administration has been seen to significantly improve insulin sensitivity (Shimura et al, 1997). Studies have also found that PPARs play an important role in glucose metabolism (Wakutsu et al, 2010). Obese mice fed fish oil showed an improvement in glucose tolerance, and by administrating PPARs antagonists, glucose levels significantly increased (Wakutsu et al, 2010). A later study in PPARγ transgenic

mice, showed that fish oil supplemented diet significantly reduced blood glucose concentration by enhancing glucose metabolism genes such as GLUT-4 in the skeletal muscles (Yu et al, 2011). As n-3 PUFAs are known ligand for PPARα (Xu et al, 1999), in rats' insulinoma cells, the expression of PPARα and RXR increased fatty acid uptake and oxidation and improved glucose-stimulated insulin secretion (Terauchi & Kadowaki, 2005). However, the effect of EPA and DHA on humans is largely controversial and needs to be further investigated. As far as could be ascertained, no studies have compared the effects of supplementation with EPA *versus.* DHA on adiponectin concentrations. This study compared the effects of supplementation with DHA versus EPA (provided as fish oil concentrates) on adiponectin concentrations compared with a placebo (oleic acid, provided as refined olive oil) in healthy subjects.

Hypothesis: DHA but not EPA will increase serum adiponectin concentrations.

7.2 Study design and participants

7.2.1 Study design

A randomised, single-blind parallel study design was used to compare three treatments. The participants were healthy men (n = 48) who were randomly allocated to consume either and EPA (3g/d) rich concentrate, DHA (3g/d) rich concentration or placebo (equivalent volume of olive oil, British Pharmacopoeia specification). The primary outcome variable was a change in serum adiponectin concentration. Secondary outcomes included insulin sensitivity (HOMA-IR) and cardiovascular risk factors (lipid profile and blood pressure). The study protocol was reviewed and approved by the Bromley Research Ethics Committee 08/H0805/2.

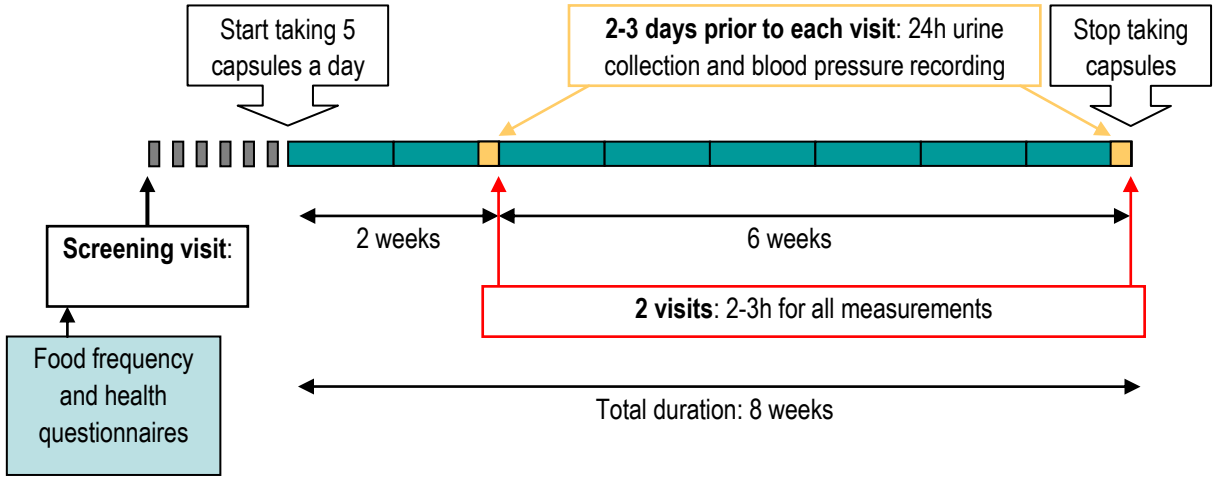

Figure 7.1 Outline of study protocol

Each treatment comprised a 6-week intervention phase. Subjects were allocated to treatment using a randomisation plan from http://www.randomization.com. A parallel design has been chosen because of the duration of the intervention and the length of time taken for cell membrane fatty acid composition to return to baseline proportions following cessation of supplementation (approximately 3-4 months); a 6-week treatment period has been chosen since previous studies have indicated that this is a sufficient length of time to see significant changes in erythrocyte membrane composition (Sanders & Younger, 1981). In order to standardise intakes prior to the beginning of the study, the subjects underwent a 2-week run-in taking placebo capsules and avoiding the consumption of oily fish, fish oil supplements or other dietary supplements (restriction of oily fish and supplements was continued for the rest of the study). Thus, the total study time for each subject was 8 weeks.

Following the run-in period, participants were asked to attend the metabolic unit on 2 occasions for 2-3 hour study visits, separated by 6 weeks, during which time they took capsules containing their allocated treatment. Unused capsules returned by participants were counted and recorded. Compliance to the dietary intervention was assessed from capsule counts and by measuring changes in the proportions of EPA and DHA in erythrocyte lipids.

7.2.2 Experimental capsules

The experimental oils, 1 g of DHA, 0.5 g of EPA and 1 g of olive oil, were supplied by Croda Europe (Hull) and encapsulated by Power Health (Pocklington, UK). EPA, DHA rich oil and olive oil were provided in the form of triglycerides in soft gel capsules. Both EPA and DHA rich oil EPA and DHA were blended with olive oil, to at least 65% content of total n-3 PUFA, to provide 0.5 g of EPA and 1 g of DHA. EPA rich oil typically contains 58.4% of EPA and DHA rich oils contain 57.5% of DHA as shown in **Table 7.1**. Subjects who were assigned to the DHA or placebo treatment were asked to consume 5 capsules a day (1g/capsule), while those who were assigned to the EPA treatment were asked to consume 10 capsules per day (0.5g/capsule). Capsules were stored at room temperature in a dry, dark room throughout the study. They were also packed in containers providing a 2-week supply for the run-in period (85 capsules) and a 6-week supply for the treatment (225 capsules for DHA or the placebo, and 450 capsules for EPA). Three days' extra capsules were added in the run-in and intervention periods and subjects were asked to bring the container back with the extra capsules after this time. The extra capsules were then counted and recorded to measure compliance. **Table 7.2** shows the fatty acid analysis of the capsules, as certified by the manufacturer. The EPA and DHA blends consisted of blends of an EPA concentrate (TG7010, code SF06396, batch 213000) and a DHA concentrate (DHA700TG, code SF06405, batch 213003) respectively and refined olive oil (batch 10813)

Table 7.1 Composition of the dietary supplements and intakes provided with an intake of 5g/d

Treatment		Composition (mg/g)		Daily intake (g/d)	
	Fat	DHA	EPA	DHA	EPA
DHA	956	575	83	2.9	0.4
EPA	956	584	88	2.9	0.4
Placebo	956	0	0	0	0

Table 7. 2 Fatty acids analysis of the capsules as certified by the manufacturer

DHA		EPA		Refined olive oil	
Fatty Acid	Content (%)	Fatty Acid	Content (%)	Fatty Acid	Content (%)
C16:0	0.7	C14:0	0.5	16:00	10.9
C16:1	0.3	C16:0	0.9	16:01	0.7
C16:2	0.5	C16:1	0.7	16:02	Trace
C16:3	0.1	C16:2	0.7	16:03	Trace
C18:0	3.7	C16:3	0.5	18:00	3.2
C18:1	6.9	C16:4	0.5	18:01	79.1
C18:2	1.6	C18:0	0.4	18:02	4.4
C18:3 n-6	0.5	C18:1	3.7	18:3n-3	0.5
C18:3 n-3	0.2	C18:2	2.1	18:4n-3	-
C18:4 n-3	0.3	C18:3 n-6	0.6	20:00	0.4
C20:0	0.6	C18:3 n-3	1.1	20:01	0.2
C20:1	2.2	C18:4 n-3	5.6	20:4n	-
C20:2 n-6	0.5	C20:0	0.5	20:5 n-3 (EPA)	-
C20:3 n-6	0.2	C20:1	0.2	22:01	-
C20:4 n-6	2.8	C20:2 n-6	0.8	22:5n-3	-
C20:4 n-3	0.7	C20:3 n-6	0.6	22:6n-3 (DHA)	-
C22:0	0.3	C20:4 n-6	4.1	Others	0.6
C20:5 n-3 EPA	8.3	C20:4 n-3	3.2	Total	100
C22:1	0.9	C20:5 n-3 EPA	58.4	Total EPA+DHA wt%	0
C21:5 n-3	0.5	C22:1	1.7	EPA + DHA g/d	0
C22:4 n-6	0.6	C21:5 n-3	1.6	Target	0
C22:5 n-6	4.6	C22:5 n-6	0.4		
C24:0	0.4	C22:5 n-3	1.5		
C22:5 n-3	3.1	C22:6 n-3 DHA	8.8		
C24:1	1.3	Others	0.9		
C22:6 n-3 DHA	57.5				
Others	0.7				

7.2.3 Participants and recruitment

Healthy males, non-smokers, aged 18-45 years, were recruited from among staff and students at King's College, London, UK by a circular email (approved by the Bromley Research Ethics Committee) and posters among King's College London students and staff. Prior to entering the main study, participants were screened to ensure eligibility.

7.2.3.1 Exclusion criteria*:*

1. Reported history of CVD (myocardial infarction; angina, venous thrombosis; stroke, and dyslipidaemia); diabetes (or fasting glucose ≥ 6.1 mmol/L); cancer, and kidney, liver, or bowel disease.
2. Presence of gastrointestinal disorder or use of a drug likely to alter gastrointestinal motility or nutrient absorption.
3. Current smokers; history of substance abuse or alcoholism (previous weekly alcohol intake >60 units/men or 50 units/women); current self-reported weekly alcohol intake exceeding 28 units.
4. Recent use of hypolipidaemic, antihypertensive, antiplatelet or antithrombotic medication.
5. Platelet count above or below the normal range, or any history indicative of a congenital or acquired platelet, or haemostatic defect.
6. Allergy or intolerance to any component of the study capsules.
7. Unwillingness to restrict consumption of any source of fish oil for the length of the study.
8. Subjects reporting consumption of >1 portion of oily fish per week.
9. Weight change of >3kg in preceding 2 months; BMI <18 and >32 kg/m2.
10. Blood pressure>160/90mmHg.
11. Fasting blood cholesterol > 6.5 mmol/L and fasting TAG concentrations > 2.0 mmol/L.

Volunteers were initially sent an email explaining the study (**Appendix 7.1**). Those who responded were initially interviewed via a questionnaire (**Appendix 7.2**), administered either over the telephone or via email, to assess whether they were suitable for the study. Volunteers who met the inclusion criteria were then invited to attend a screening session. We anticipated that it would be necessary to invite ~80 people for the screening visit to get 60 subjects recruited onto the study, allowing for 10 drop-outs to achieve a final sample size of 50 subjects. We also included a run-in period which was meant to help exclude subjects unlikely to comply with the intervention.

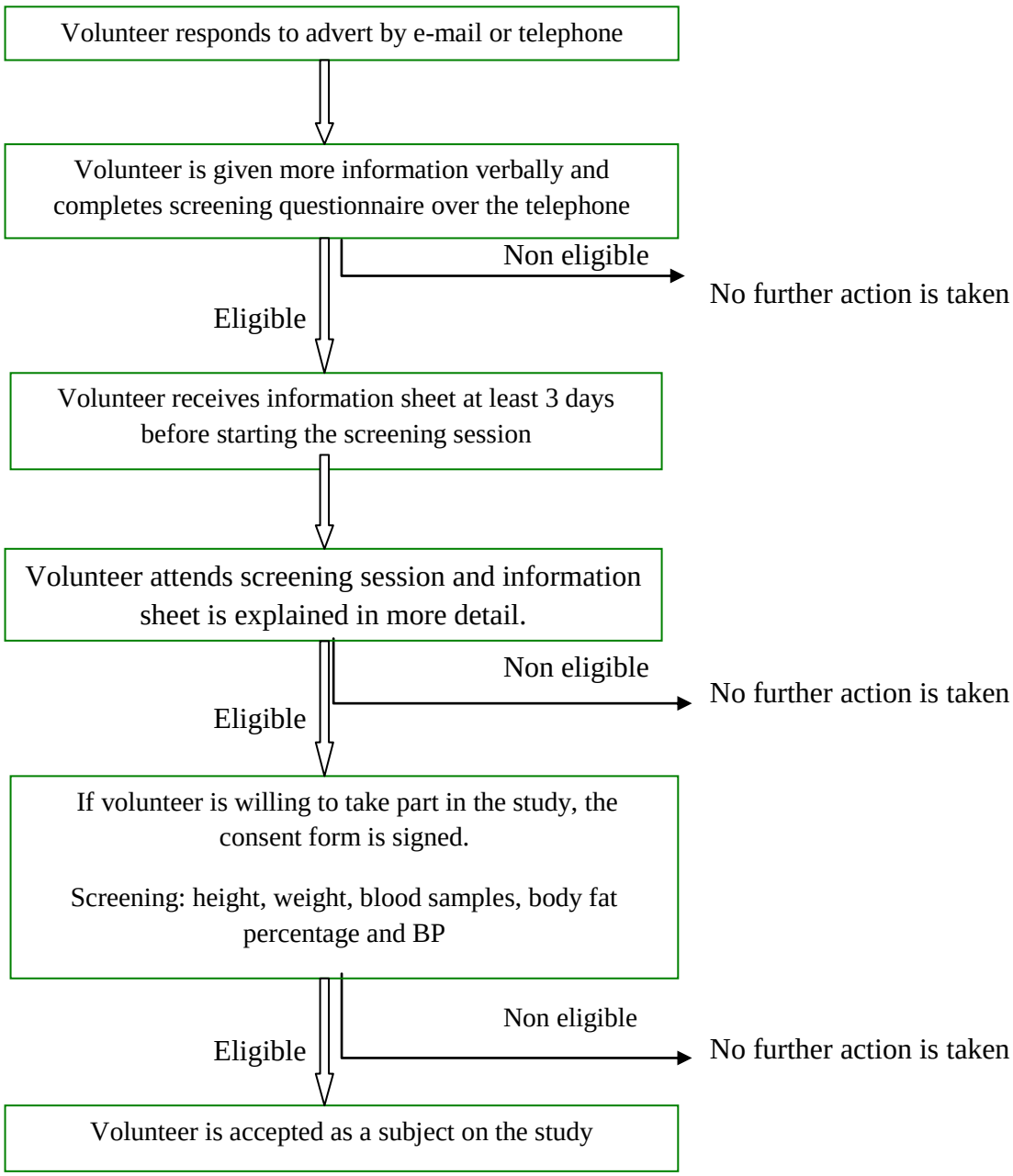

Figure 7.2 Methodology of recruitment

7.3 Equipment, consumables and supplements

7.3.1 List of equipment:

Item	Supplier
Centrifuge Jouan CR4.12 for blood handling	DJB Labcare Ltd., Buckinghamshire, UK
Calibrated automated BP monitor	Omron 7051T, Omron Healthcare Europe BV
Bioelectrical impedance analysis equipment	BC-418 MA, Tanita UK Ltd., Middlesex, UK
Ambulatory BP	TM-3430, A&D Instrument Ltd, Abingdon, UK
Digital Volume Pulse	DVP; Pulse Trace PCA 2, Micro Medical Ltd
Pulse Wave analysis	PWA; SphygomoCor Px, AtCor Medical Pty Limited

7.3.2 Consumables (Venous blood collection tubes):

Item	Supplier
4.5 mL EDTA lavender tubes	Becton Dickinson; cat.no. 367654, UK
4 mL fluoride/oxalate (FX) grey tubes	Becton Dickinson; cat.no. 367922, UK
8.5 mL gold tubes with clot activator and gel for serum separation	Becton Dickinson; cat no. 367953, UK
6 mL gold tubes with clot activator and gel for serum separation	Becton Dickinson; cat no. 367953, UK
4 mL lithium heparin (LH) green tubes	Becton Dickinson; cat no. 367884, UK

7.3.3 Supplements

Item	Supplier
0.5g EPA-rich oil soft gel capsules	Incromega EPA500TG SR, Croda Chemicals Europe Ltd, Goole, UK
1g DHA-rich oil soft gel capsules	Incromega EPA500TG SR, Croda Chemicals Europe Ltd, Goole, UK
1g olive oil soft gel capsules	Incromega EPA500TG SR, Croda Chemicals Europe Ltd, Goole, UK

7.4 Screening procedure

Potential participants arrived between 8.30 and 10.00 am and were briefed on what the study involved. They were provided with a copy of the 'Information sheet for participants' **(Appendix 7.3)**. A more detailed study booklet was also provided to them with additional information regarding study dates **(Appendix 7.4)**. The participants were provided with a list of oily fish to avoid during the 8 weeks of intervention **(Appendix 7.5)**. They were then required to sign the consent form **(Appendix 7.6)**. All required information was documented on a standard screening record sheet. After consent had been obtained, waist and hip circumferences were measured to the nearest 0.1 cm using a plastic tape measure. Height was measured using a stadiometer. Percentage body fat was estimated using bioelectrical impedance and seated BP was measured with the OMRON 705 CPII auto upper arm BP monitor. In addition, a fasting venous blood sample was obtained.

Participants were requested to select an 8-week regime, during which they were able to take 5 or 10 capsules a day and attend the metabolic unit twice for a 2-hour visit after 2 and 8 weeks of intervention. A light breakfast was provided for the participants before leaving the screening session.

7.4.1 Screening samples and blood handling

Following the 10-12 hour overnight fast, venous blood samples were collected by Miss Sarah Cottin using butterfly needles into evacuated tubes with the minimum compression necessary to display the vein. Standard procedures are discussed in **Appendix 7.7**, according to the blood collection protocol outlined in **Appendix 7.8**. Miss Cottin is a qualified phlebotomist. Blood samples were handled according to the protocols.

Blood was screened for haematology, glucose, lipid profile and liver function. Blood for haematology was collected into 4.5 mL tubes containing EDTA, for plasma glucose, into 4mL fluoride/oxalate grey tubes and for serum lipids and liver function, into a 6 mL tube with no anticoagulant. All apart from the EDTA tube (whole blood) were then centrifuged at 3000 rpm for 15 min at 2-4º C and samples were then dispatched by motorcycle courier on the day of collection for same day analysis to Dr. Roy Sherwood, Department of Clinical Biochemistry at King's College Hospital, London, which is a

Clinical Pathology Accreditation Ltd. (CPA) laboratory (CPA 1245). Full blood counts were analysed through routine chemistry using a SYSMEX counter (Sysmex UK Ltd., Milton Keynes, UK). Plasma glucose was determined by the glucose oxidase method on the ADVIA 1650 automated chemistry analyser (Bayer Diagnostics). Assays for liver function and lipids were carried out using the ADVIA 1650 automated chemistry analyser (Bayer Diagnostics, Berkshire, UK). Total protein was measured using the Biuret reaction; albumin with bromocresol green; bilirubin through sulphanilic acid diazotisation; alkaline phosphatase using p-nitrophenol phosphate as substrate; aspartate aminotransferase using aspartate to glutamate conversion, monitored by UV, and gamma glutamyl transpeptidase, using γ-glutamyl-4 nitroanilide as a substrate. TC concentrations were measured using cholesterol oxidase and 4-aminoantipyrene; TAG was measured using lipoprotein lipase, and 4-aminoantipyrine. HDL-C was measured as for TC after the addition of an antibody, which binds LDL-C, VLDL and IDL cholesterol. LDL-C was calculated using the Friedewald formula (LDL-C = TC – HDL-C – TAG / 2.2; in mmol/L) (Friedewald et al, 1972).

7.5 Instructions prior to visits

Three to four days before each visit, participants were asked to attend the metabolic unit for short visits (20-30 minutes). On this visit, the participants were shown how to use the BP monitor and instructed on the 24-hour urine collection. Written instructions for both procedures and a diary card, where the subject was asked to record his activities during the 24- hour BP monitoring, were provided (**Appendix 7.9 and 7.10**). Two to three days prior to each visit, participants were asked to record their BP and collect their urine for 24 hours.

The day prior to the visit, participants were asked to avoid certain foods/drinks and activities due to their effects on vascular function and BP, i.e. foods high in fat; alcohol; caffeine from midday (Hartley et al, 2004), and vigorous exercise. They were also asked to refrain from oily fish consumption for 2 weeks prior to the first visit. They were further asked to fast overnight and instructed to avoid eating or drinking anything, except water, for 12 hours before the time of their scheduled study visit and to avoid exercise on the morning of the study. An emailed reminder (**Appendix 7.11**) was sent out on the morning before the study day, summarising all instructions.

7.6 On the study day:

A maximum of 2 subjects were booked each day. Participants arrived between 8 and 11 am the following morning. The subjects' record sheet was completed (**Appendix 7.12)** their weight, body composition and seated BP were measured; a 24-hour urine sample and the 24-hour BP monitor reading were collected. Subjects were allowed to rest quietly in a supine position for 15 minutes prior to the start of vascular measurements. Temperature was recorded before and after measurements, all of which were carried out on the left arm, while BP was measured on the right arm. Finally, a fasting blood sample was collected.

7.6.1 Anthropometry:

Height was measured to the nearest 0.1 cm using a wall-mounted stadiometer. waist circumference was measured to the nearest 0.1 cm in the standing position with arms to the side, shoulder-width apart, and weight equally distributed on each leg. The measurements were standardised as the mid-point between the lower rib margin and the iliac crest. Where possible, measurements of participants were taken with their waists uncovered or else without heavy outer garments. Participants were asked to breathe normally and the reading was taken at the end of a normal exhalation. Body composition was assessed using a Tanita BC-418 segmental body composition analyser.

7.6.2 Seated BP

BP and heart rate were taken using an OMRON 705CPII, or equivalent, auto upper arm BP monitor. These measurements were taken while the participant was seated and after 10 minutes of rest. When taking a BP measurement, the volunteer was calm and relaxed, sitting quietly with legs uncrossed and feet flat on the floor. At the same time, the arm from which the measurement was being taken was supported. A cuff of appropriate size was selected and wrapped snugly around the upper arm with space for a finger between the volunteer and cuff. The cuff was also appropriately aligned with the brachial artery, following the instructions contained in the equipment manual. Measurements of systolic and diastolic BP, as well as heart rate were made. The BP measurements were repeated at least three times at two-minute intervals. The value for the first reading was discarded and the mean of the two following readings was taken according to the British Hypertension Society Guidelines (O'Brien & Staessen, 2000).

7.6.3 Ambulatory BP (to be used for another study)

A&D TM-2430 ambulatory blood pressure (ABP) monitoring devices were used. These have been given A/A grading and have been approved for clinical use by the British Hypertension Society (Williams et al, 2004). Measurements were made in accordance with British Hypertension Society guidelines. The participants were shown how to use the ABP monitors. Triplicate measurements of clinic-based BP using an automated BP monitor were taken on the right and left arm, to ensure there were no differences in BP between arms. Participants were then provided with a suitably sized cuff (small, medium or large), as well as a brief instruction card and a diary card. The diary card was completed by the participant following each measurement, in order for them to report what activity they were doing immediately prior to the measurement. The ABP was programmed to take measurements every 30 minutes during the day (0700 h -22:00 h and hourly at night (22:00 h-07:00 h). The data was analysed using TM-2430-13 Doctor Pro Software and checked for accuracy. The first 3 readings following fitting the monitor were discarded. A minimum of 24 awake and 4 night-time readings was required. If unsatisfactory readings were obtained, the participant was requested to repeat the 24-hour BP monitoring.

7.6.4 Vascular functions (to be used for another study)

Peripheral arterial tone (DVP) was measured by using the forefinger on the radial pulse to obtain a reflection index (RI), stiffness index (SI), and pulse wave analysis (PWA), to obtain the peripheral augmentation index (PAIx). Supine BP was measured concurrently. These vascular measures were made in triplicate, according to the following sequence: (1) a single DVP measured on the left index finger, followed by (2) a BP measurement of the right brachial artery using a calibrated automated BP monitor, and finally (3) a PWA carried out on the left radial artery. Vascular functions were conducted by the author and Miss Sarah Cottin. At the end of the visit, blood was collected. The detailed steps of DVP and PWA are outlined in **Appendix 7.13.**

7.6.5 Blood sample collection, handling and analysis

Venous blood samples for the analysis of plasma glucose, insulin, TAG, TC, LDL-C, HDL-C, Apo B100, NEFA, adiponectin were collected into evacuated tubes with the minimal compression necessary to display the vein. The blood for glucose analysis was collected into 4 ml fluoride oxalate. Blood for insulin analysis was collected into 4 ml lithium heparin tubes and blood for serum TAG, TC, LDL-C, HDL-C, Apo -B100, and NEFA was collected into 8.5 mL serum tubes, containing a clot activator and gel for serum separation. Blood for serum adiponectin was also collected into 8.5 mL serum tubes containing a clot activator and gel for serum separation. Tubes were then centrifuged at 3000 rpm for 15 minutes at 4ºC and plasma samples, glucose and insulin were stored at -40ºC until analysis, with serum samples being stored at -80^{o}C until analysis.

At the end of the 2-hour visit, the participants were provided with a light meal to consume before leaving the metabolic unit.

7.7 Determination of measured variables

7.7.1 Determination of plasma glucose*:*

Determination of plasma glucose, insulin, TAG, TC, LDL-C, HDL-C, Apo B100, NEFA and adiponectin were analysed by the author in the Clinical Chemistry laboratories at King's College Hospital with the guidance from Tracy Dew.

Sample requirements

The sample was taken into a fluoride-oxalate tube. The ADVIA 2400 used 3.4 µl of sample in the test.

Method

Glucose reagent was supplied by Siemens Healthcare Diagnostics Ltd, Newton House, Sir William Siemens Square, Frimley, Camberley, Surrey, GU16 8QD.

Glucose in the sample was phosphorylated by the transfer of phosphate from adenosine triphophosphate (ATP), via the action of hexokinase. Glucose-6-phosphate formed in this reaction was oxidised by glucose-6-phosphate dehydrogenase. This was accompanied by the reduction of NAD+ to NADH, which resulted in an increase in absorbance at 340 nm, proportional to the glucose concentration in the sample. The increase in absorbance was converted to glucose concentration by reference to a

previously determined calibrator and reagent blank. The effect of any interfering substances was reduced by blanking each sample using reagent 1 (buffer and co-factors) before the addition of reagent 2 (enzymes). Inter-assay CV was 1.6% and intra-assay CV was 0.6 %.

7.7.2 Determination of plasma insulin*:*

Sample requirements

To assay the sample in duplicate, 50 μL was used. However, the sample tube contained a volume of 250 μL to account for dead volume and dilution.

Method

Insulin reagent supplied by Siemens Healthcare Diagnostics Ltd, Newton House, Sir William Siemens Square, Frimley, Camberley, Surrey, GU16 8QD.

The Siemens Advia Centaur assay is a two-site sandwich immunoassay using direct chemiluminometric technology, through the application of constant amounts of two antibodies. The sample was incubated with two insulin-specific antibodies. The first was in the Lite Reagent, which is a monoclonal mouse anti-insulin antibody labelled with acridinium ester. The second antibody, in the solid phase, is a monoclonal mouse anti-insulin antibody, which was covalently coupled to paramagnetic particles. Insulin formed a sandwich between the two antibodies. After incubation, a magnetic field was applied, bringing about the solid phase (including the sandwich) held at the site of the reaction cuvette, while the liquid phase was aspirated. The cuvette was then washed with deionised water. Next, acid reagent (containing hydrogen peroxide) was added to the cuvette to begin a light emission reaction with the acridinium ester. The cuvette was then moved to the luminometer and base reagent was added to enhance the light reaction. Light intensity was measured immediately and converted to relative light units. This had a direct proportional relationship with insulin concentration. Inter-assay CV was 5.9% and intra-assay CV was 4.6 %.

7.7.3 Determination of serum cholesterol*:*

Sample requirements

Serum sample was used. The ADVIA 2400 used 2 µl of sample in the test.

Method

Cholesterol reagent supplied by Siemens Healthcare Diagnostics Ltd, Newton House, Sir William Siemens Square, Frimley, Camberley, Surrey, GU16 8QD.

Serum cholesterol was determined using an enzymatic method. Cholesterol esterase completely hydrolyses cholesterol esters in serum to free cholesterol, which is in turn oxidised by cholesterol oxidase to generate hydrogen peroxide. The hydrogen peroxide formed combines with 4-aminophenazone and a phenol to form a red quinone amine dye, measured as an endpoint reaction at 505/694 nm. The increase in dye absorbance here was directly proportional to the concentration of cholesterol in the sample, when compared to a previous calibration assay. Inter-assay CV was 1.1% and intra-assay CV was 0.6 %.

7.7.4 Determination of HDL-C*:*

Sample requirements

The ADVIA 2400 used 2 µl of sample in the test.

Method

HDL–C reagents supplied by Siemens Healthcare Diagnostics Ltd, Newton House, Sir William Siemens Square, Frimley, Camberley, Surrey, GU16 8QD.

The Siemens Advia Direct HDL-C method is a two-step automated procedure. In the first step, cholesterol esterase and cholesterol oxidase react to remove non-HDL-C from the sample. The hydrogen peroxide produced is then removed by the enzyme catalase. The absence of detergent in this first reaction prevents HDL-C from reacting with the enzymes. In Stage 2, detergent is added to allow HDL-C to react with the enzyme system. Sodium azide inhibits the reaction of the hydrogen peroxide formed with catalase. The hydrogen peroxide acts with 4-aninoantipyrine to produce a quinoneimine pigment measured at 596 nm. Here, the inter-assay CV was 2.2% and intra-assay CV was 1.1 %.

7.7.5 Determination of serum Apo B:

Sample requirements The ADVIA 2400 used 2 µl of sample in the test.
Method Apo B reagent supplied by Siemens Healthcare Diagnostics Ltd, Newton House, Sir William Siemens Square, Frimley, Camberley, Surrey, GU16 8QD.

The apolipoprotein method is a polyethylene glycol enhanced immunoturbidimetric assay. A sample containing human Apo B and specific antiserum was found to form an insoluble complex measured turbidimetrically at 340/694nm. By constructing a standard curve from the absorbance of standards, the concentration of Apo B was determined. Inter-assay CV was 3.3% and intra-assay CV was 1.6 %.

7.7.6 Determination of serum NEFA:

Method
NEFA Assay kit is supplied by WAKO Chemicals Gmbh, Fuggerstrasse 12, D-41468 Neuss, Germany.
In the sample, NEFA was converted to acyl coenzyme A, AMP and pyrophosphoric acid by the action of ACS, under coexistence with coenzyme A (CoA) and ATP. The acyl coenzyme A obtained was oxidised and yielded 2,3-trans-Enoyl-CoA and hydrogen peroxide through the action of acyl coenzyme A oxidase In the presence of peroxide, the hydrogen peroxide formed yielded a blue-purple pigment via quantitative oxidation condensation with 3-Methyl-N-Ethyl-N-(ß-Hydroxyethyl)-Aniline (MEHA) and 4-amino-antipyrine (4-AA). Non-esterified fatty acid concentration was obtained by measuring the absorbance of the blue-purple colour. Inter-assay CV was <1.5% and intra-assay CV was <1.5 %.

7.7.7 Determination of serum TAG

Sample requirements The ADVIA 2400 used 2 µL of sample in the test.
Method TAG reagents supplied by Siemens Healthcare Diagnostics Ltd, Newton House, Sir William Siemens Square, Frimley, Camberley, Surrey, GU16 8QD.
The Siemens Advia method for the measurement of TAG is an enzymatic assay. TAGs are converted to glycerol and free fatty acids by lipase. Here, the glycerol was then converted to glycerol-3-phosphate by glycerol kinase, followed by its conversion through the action of glycerol-3-phosphate-oxidase to hydrogen peroxide. A coloured complex was formed from hydrogen peroxide, 4-aminophenazone and 4-chlorophenol

under the catalytic influence of peroxidase. The absorbance of the complex was measured as an endpoint reaction at 505/694 nm. Inter-assay CV was 0.033% and intra-assay CV was 0.6 %.

7.7.8 Determination of serum adiponectin

Sample requirements

To assay the sample in duplicate, a 50 μL was required. However, the sample tube should contain at least a volume of 250 μL to account for dead volume and dilution. All samples were pre-diluted 1/100 before being assayed.

Method

Kit used was Quantikine Adiponectin ELISA distributed by R & D Systems Europe, 19 Barton Lane, Abingdon Science Park, Abingdon, Oxon, OX14 3NB.

This assay contains NSO-expressed recombinant human adiponectin and has been shown to accurately quantitate the recombinant factor. This assay measures total (low, middle, and high molecular weight) adiponectin.

This assay employs the quantitative sandwich enzyme immunoassay technique. A monoclonal antibody specific for adiponectin was pre-coated onto a microplate. Standards and samples were pipetted into the wells and any adiponectin present was bound by the immobilised antibody. After washing away any unbound substances, an enzyme-linked monoclonal antibody specific for adiponectin was added to the wells. Following a wash to remove any unbound antibody-enzyme reagent, a substrate solution was added to the wells and colour developed in proportion to the amount of adiponectin bound in the initial step. The colour development was stopped and the colour intensity measured. Inter-assay CV was 6.8% and intra-assay CV was 2.5%.

Linearity

Any results above the concentration 250.0 ng/mL were reported as >250. If necessary, the samples can be diluted at 1:5 using the appropriate calibrator diluent and repeated in the subsequent batch. The results obtained from the assay were multiplied by the original dilution factor of 100.

7.7.9 Erythrocyte lipid fatty acid composition

Erythrocyte membrane phospholipid fatty acid composition was conducted by Miss Sarah Cottin. For detailed methodology refer to **Appendix 7.14.**

7.8 Statistical analyses

Power calculations were conducted using adiponectin as the primary endpoint. Using StatMate (GraphPad.com) gave 80% power at $P < 0.05$ to detect a 1 SD unit difference in adiponectin between treatments. Therefore, the study aimed to recruit a total of 48 subjects. Data were tested for normality and analysed on an intention to treat basis using SPSS version 17.0 (SPSS Inc., Chicago, IL). Data were log-transformed for plasma TAG, HDL-C, adiponectin, insulin and HOMA-IR to render their distribution normal before statistical analysis. Baseline, follow-up means, and mean changes from baseline were analysed using the ANCOVA model, with treatment as a factor, follow-up values as dependant variable, and baseline values, age, BMI and ethnicity as covariates. All data presented in text and tables are expressed as means or geometric means ± SD or 95% CI.

7.9 Results

7.9.1 Subjects

Recruitment onto the trial commenced in June 2009 and was completed in May 2010. In response to the recruitment campaigns, an approximate total of 70 people expressed an interest in the study and were provided with the study information sheet. From those, 57 individuals expressed further interest and were interviewed using the telephone questionnaire. Of these, one subject did not meet the inclusion criteria. Six decided not to participate, and were excluded after the screening visit prior to randomisation. Thus, 12.3% (*n* = 7) of potential participants were excluded during the recruitment screening process leaving 49 who were randomised to treatment. Out of these, (*n* = 1) dropped out of the study. As shown in **Figure 7.3.**

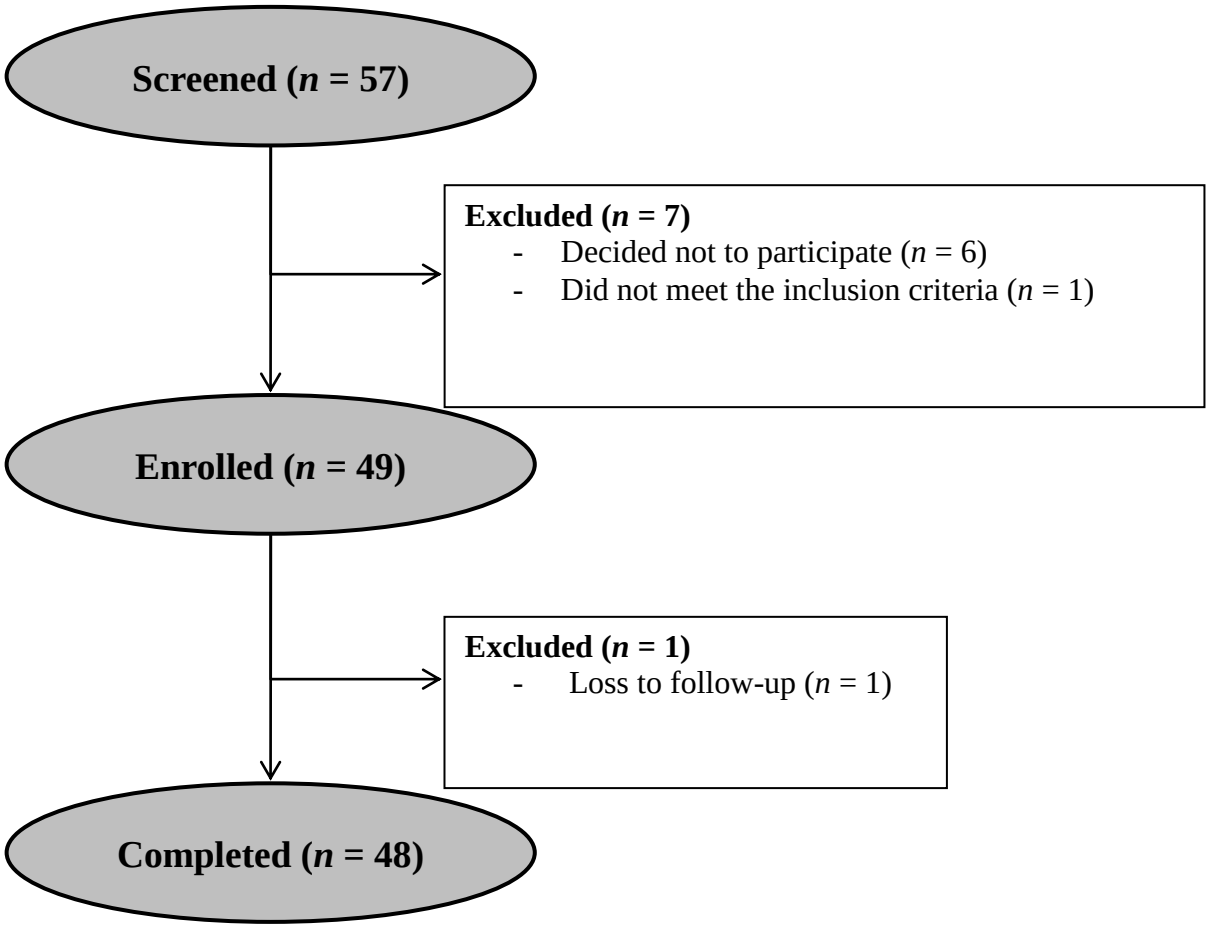

Figure 7.3 The Consort Flow Chart

7.9.2 Baseline characteristics of subjects

A total of 48 subjects completed the study. The ethnic mix of subjects was predominantly White. In fact, White subjects made up 62.5% (*n* = 30), Black Africans: 10.4% (*n* = 5), Indians and Middle-Eastern individuals: 16.7% (*n* = 8), and Chinese and East Asians: 10.4 % (*n* = 5). Their characteristics at recruitment with respect to treatment allocation are presented in **Table 7.3**. There were no significant differences in measured variables between subjects in treatment allocated groups. Fasting glucose was slightly higher in the DHA group compared to the EPA and olive oil groups. The average BMI was within the desirable range (20-25 kg/m^2) and the mean waist circumference were lower than the cut-offs used to indicate risk of metabolic syndrome (94 cm in men). Body weights were stable and there were no differences in changes in BMI, body fat percentage, systolic and diastolic BP, or heart rate between treatments, as shown in **Table 7.4**.

Table 7. 3 Characteristics of subjects at baseline by randomised treatment

	Treatment group		
Phenotype	**Olive oil (*n* = 16)**	**DHA (*n* = 16)**	**EPA (*n* = 16)**
Age (years)	25.8 ±7.4	27.9 ±8.0	25.8 ±7.5
Clinic Systolic BP (mm Hg)	119 ±10	125 ±10	119 ±14
Clinic Diastolic BP(mm Hg)	67 ±6	72 ±8	68 ±12
Heart rate (beats/min)	59 ±6	66 ±10	67 ±10
Height (m)	1.76 ±0.07	1.75 ±0.08	1.79 ±0.08
Weight (kg)	73.0 ±12.2	72.1 ±12.5	73.5 ±11.2
BMI (kg/m^2)	23.6 ±3.4	23.3 ±2.9	22.9 ±3.0
Waist (cm)	81.7 ±10.2	82.5 ±10.3	80.6 ±8.3
Hip circumference (cm)	99.3 ±8.7	99.8 ±8.6	98.7 ±8.0
Waist:/Hip ratio	0.8 ±0.0	0.8 ±0.1	0.8 ±0.1
Body fat (%)	16.6 ±5.5	14.4 ±4.5	13.5 ±4.5
Fat mass (kg)	12.5 ±6.1	10.8 ±4.9	10.2 ±4.0
Plasma glucose[1]	4.9± 0.3	5.3± 0.4	5.1± 0.3
TC (mmol/L)	4.6 ± 0.6	4.4 ± 0.7	4.5 ± 0.7
TAG (mmol/L)[2]	0.8 ± 0.3	0.8 ± 0.3	0.9 ± 0.5
HDL-C (mmol/L)[2]	1.4 ± 0.4	1.3 ± 0.3	1.3 ± 0.2
LDL-C(mmol/L)	2.7 ± 0.5	2.7 ± 0.6	2.7± 0.6
TC:HDL-C ratio	3.2 ± 0.7	3.5 ± 0.8	3.4 ± 0.8

Measurements made at the allocation of treatment: placebo, DHA or EPA. Values are mean ± SD and do not differ by treatment allocation. Association was tested by ANOVA [1]Significant difference between groups. [2]Geometric means.

Table 7. 4 BMI, body fat %, clinic BP and heart rate at baseline and after 6 weeks of supplementation with the placebo, DHA or EPA.

	Olive oil (*n* =16) (Mean, 95% CI)	**DHA (*n* = 16)** (Mean, 95% CI)	**EPA (*n* = 16)** (Mean, 95% CI)	*P* value
BMI (kg/m^2)[1]				
Baseline	23.5 (21.3, 25.7)	21.1 (16.8, 25.3)	21.5 (18.6, 24.5)	
Follow-up	23.4 (21.3, 25.6)	23.6 (21.8, 25.4)	21.8 (19.1, 24.5)	
Change	0.0 (-0.2, 0.2)	0.7 (-0.4, 1.9)	0.3 (-0.2, 0.7)	0.26
Treatment effect	0.000 [Reference]	0.69 (-1.6, 0.2)	0.06 (-1.1, 1.0)	
Body fat %[1]				
Baseline	16.6 (13.8, 19.4)	15.0 (12.8, 17.1)	13.7 (11.3, 16.1)	
Follow-up	16.5 (13.6, 19.3)	14.2 (12.2, 16.2)	13.9 (11.5, 16.3)	
Change	-0.2 (-1.2, 0.9)	-0.5 (-1.9, 0.9)	0.2 (-0.8, 1.2)	0.70
Treatment effect	0.000 [Reference]	-0.58 (-0.9, 2.1)	0.01 (-1.5, 1.5)	
Systolic BP (mm Hg)				
Baseline	121 (116, 125)	122 (117, 126)	118 (113, 122)	
Follow-up	119 (114, 124)	121 (115, 126)	116 (110, 121)	
Change	-2.0 (-5.6, 1.6)	-1.1 (-4.7, 2.5)	-2.3 (-5.9, 1.3)	0.88
Treatment effect	0.000 [Reference]	1.05 (-4.0, 6.1)	-0.69 (-5.8, 4.4)	
Diastolic BP (mm Hg)				
Baseline	67 (63, 71)	69 (65, 74)	69 (64, 73)	
Follow-up	66 (61, 70)	68 (64, 73)	66 (61, 70)	
Change	-1.4 (-4.2, 1.5)	-1.1 (-3.9, 1.8)	-2.7 (-5.5, 0.1)	0.86
Treatment effect	0.000 [Reference]	0.70 (-3.2, 4.6)	-1.04 (-5.0, 2.9)	
Heart rate (beats/min)				
Baseline	63 (58, 69)	66 (61, 72)	66 (60, 71)	
Follow-up	65 (59, 70)	65 (60, 71)	65 (60, 71)	
Change	1.4 (-4.3, 7.2)	-0.9 (-6.7, 4.8)	-0.6 (-6.3, 5.2)	0.99
Treatment effect	0.000 [Reference]	0.80 (-6.2, 7.8)	0.67 (-6.3, 7.6)	

Measurements made at baseline after a 2-week run-in with placebo supplements and after 6 weeks with placebo, DHA or EPA supplements at daily doses of 3 g. Association was tested by ANCOVA with baseline values as covariate. *P* values are adjusted for age, BMI and ethnicity. Values are means (95% CI). [1] *P* values are adjusted for age and ethnicity

7.9.3 Compliance with taking the supplements

Compliance to the dietary intervention was assessed from capsule counts and by measuring changes in the proportions of EPA and DHA in erythrocyte lipids. Out of the 48 subjects, 32 returned the remaining capsules. Of these a total of 3 (6.2%) were estimated to have consumed less than 90% of the capsules and were classified as poor compliers. There were no statistically significant differences in the reported capsule intakes between treatment groups. **Table 7.5** shows erythrocyte fatty acid composition (%) at baseline and after EPA, DHA or placebo 3g/d. Compared to placebo, both EPA and DHA increased total n-3 PUFA by 4.5 % (95% CI: 2.9, 6.1) and 2.0 % (95% CI: 0.5, 3.6) respectively. DHA treatment had no effect on EPA or docosapentaenoic acid (22:5n-6 DPA) DPA levels but significantly increased DHA levels by 1.7 % (95% CI: 0.6, 2.8). EPA treatment significantly increased both EPA and DPA levels by 3.0% (95% CI: 2.4, 3.7) and 1.0 % (95% CI: 0.6, 1.3) respectively. Neither EPA nor DHA affected total SFA, MUFA or PUFA levels. Compared to placebo, both EPA and DHA decreased n-6 PUFA, however, this decrease was only significant with EPA treatment -5.4% (-7.3,-3.5). EPA and DHA significantly increased omega-3 index by 3.5% (95% CI: 2.0, 5.1) and 2.1% (95% CI: 0.6, 3.7). Generally, compliance to the supplements appeared good except in one subject allocated to the DHA who had a low DHA concentration at baseline which did not increase during supplementation as expected.

Table 7. 5 Erythrocyte fatty acid composition (%) at baseline and after EPA, DHA or placebo

	Placebo		DHA		EPA		*P*
	Baseline	Follow-up	Baseline	Follow-up	Baseline	Follow-up	
Total SFA	33.0 ± 2.8	32.8 ± 3.9	31.0 ± 5.5	32.3 ± 4.9	32.8 ± 3.5	34.1 ± 2.7	0.06
16:0	17.5 ± 2.0	17.5 ± 3.1	16.1 ± 3.7	17.0 ± 3.1	17.4 ± 2.6	18.4 ± 1.6	0.15
18:0	15.6 ± 1.2	15.2 ± 1.1	14.8 ± 2.0	15.2 ± 1.9	15.4 ± 1.2	15.7 ± 1.3	0.26
Total MUFA	16.4 ± 1.0	16.7 ± 1.1	16.9 ± 1.5	16.9 ± 1.3	16.7 ± 1.2	16.2 ± 1.3	0.07
16:1	0.4 ± 0.2	0.4 ± 0.5	0.3 ± 0.1	0.3 ± 0.1	0.3 ± 0.1	0.3 ± 0.1	0.96
18:1 trans	0.3 ± 0.1	0.2 ± 0.1	0.2 ± 0.1	0.2 ± 0.1	0.2 ± 0.1	0.2 ± 0.1	0.94
18:1n-9	14.5 ± 1.0	14.8 ± 1.1	15.2 ± 1.4	15.3 ± 1.4	14.9 ± 1.2	14.6 ± 1.3	0.12
18:1n-7	1.2 ± 0.1	1.3 ± 0.1	1.2 ± 0.2	1.2 ± 0.2	1.3 ± 0.1	1.2 ± 0.1	0.08
Total PUFA	46.1 ± 2.6	46.3 ± 4.3	46.4 ± 5.2	46.8 ± 4.5	45.7 ± 4.2	45.2 ± 2.2	0.37
Total n-6 PUFA	35.8 ± 2.7	35.8 ± 4.6	35.7 ± 3.3	34.2 ± 5.1	35.6 ± 3.8	30.3 ± 3.8*	<0.001
18:2n-6	12.3 ± 1.2	12.2 ± 1.6	11.9 ± 0.8	11.3 ± 1.3	12.1 ± 1.2	10.4 ± 1.7*	0.002
20:3n-6	2.1 ± 0.4	2.3 ± 1.0	2.2 ± 0.6	2.0 ± 0.7	2.4 ± 0.7	1.7 ± 0.4*	0.006
20:4n-6	17.2 ± 1.8	17.1 ± 2.2	17.6 ± 2.4	16.9 ± 2.9	17.1 ± 2.3	15.1 ± 1.8*	0.001
22:4n-6	3.6 ± 0.7	3.6 ± 0.8	3.7 ± 1.0	3.4 ± 1.1	3.5 ± 0.9	2.7 ± 0.9*	0.002
22:5n-6	0.6 ± 0.1	0.5 ± 0.2	0.5 ± 0.1	0.6 ± 0.1	0.6 ± 0.1	0.4 ± 0.1*	<0.001
Total n-3 PUFA	10.3 ± 1.7	10.5 ± 1.5	10.7 ± 2.6	12.6 ± 2.3*	10.1 ± 2.1	14.9 ± 2.5*	<0.001
18:3n-3	0.2 ± 0.1	0.2 ± 0.1	0.2 ± 0.1	0.2 ± 0.1	0.2 ± 0.1	0.2 ± 0.1	0.61
20:5n-3	1.1 ± 0.4	1.1 ± 0.4	1.1 ± 0.5	1.6 ± 0.6	1.1 ± 0.4	4.2 ± 1.4*	<0.001
22:5n-3	3.2 ± 0.5	3.2 ± 0.6	3.4 ± 0.5	3.1 ± 0.6	3.4 ± 0.7	4.2 ± 0.4*	<0.001
22:6n-3	5.8 ± 1.3	6.0 ± 1.3	6.0 ± 1.8	7.7 ± 2.0*	5.4 ± 1.6	6.3 ± 1.2	0.01
n3 index	6.9 ± 1.5	7.1 ± 1.5	7.2 ± 2.2	9.3 ± 2.5*	6.6 ± 1.9	10.5 ± 2.4*	<0.001
ration-6:n-3	3.6 ± 0.7	3.5 ± 0.8	3.5 ± 0.7	2.8 ± 0.9*	3.7 ± 0.9	2.2 ± 0.8*	<0.001

Data is presented as mean ± SD and expressed in % of total erythrocyte fatty acid composition. Differences between the three treatments were tested by ANOVA. When comparing the follow up values to placebo, association was tested by ANCOVA with baseline values as covariate. **P*-values when nominally significant (< 0.05) when compared to placebo treatment

7.9.4 Fasting serum lipid concentrations:

Table 7.6 shows the results for fasting serum lipids and NEFA concentrations. There was a treatment effect for plasma NEFA (P = 0.05) but none of the individual comparisons between treatments achieved significance using Bonferroni's multiple comparison test. There were no other statistical significant differences. TAG values were low in all groups and showed a small decline following all treatments. Plasma Apo B concentrations showed a tendency to increase following DHA compared with the other treatments but the difference was not statistically significant.

Table 7. 6 Plasma lipids at baseline and after 6 weeks of supplementation with the placebo, DHA or EPA.

Phenotype	Treatment group			
	Olive oil (*n* = 16) (Mean, 95 % CI)	**DHA (*n* = 16)** (Mean, 95 % CI)	**EPA (*n* = 16)** (Mean, 95 % CI)	*P* value
TC (mmol/L)[1]				
Baseline	4.7 (4.3, 5.1)	4.4 (4.0, 4.8)	4.6 (4.2, 5.0)	
Follow-up	4.6 (4.2, 5.0)	4.6 (4.2, 5.0)	4.6 (4.2, 5.0)	
Change	-0.1 (-0.5, 0.2)	0.2 (-0.1, 0.5)	0.0 (-0.3, 0.3)	0.74
Treatment effect	0.000 [Reference]	0.03 (-0.1, 0.1)	0.02 (-0.1, 0.1)	
Apo B (g/L)				
Baseline	0.88 (0.77, 0.99)	0.80 (0.69, 0.92)	0.90 (0.79, 1.01)	
Follow-up	0.81 (0.70, 0.92)	0.88 (0.77, 0.99)	0.84 (0.74, 0.95)	
Change	-0.07 (-0.17, 0.04)	0.08 (-0.02, 0.18)	-0.06 (-0.16, 0.04)	0.24
Treatment effect	0.000 [Reference]	0.11 (0.0, 0.2)	0.02 (-0.1, 0.1)	
LDL-C (mmol/L)				
Baseline	2.9 (2.6, 3.2)	2.7 (2.4, 3.0)	2.8 (2.5, 3.1)	
Follow-up	2.8 (2.5, 3.1)	2.9 (2.5, 3.2)	2.8 (2.4, 3.1)	
Change	-0.1 (-0.3, 0.2)	0.2 (-0.1, 0.4)	0.0 (-0.3, 0.2)	0.38
Treatment effect	0.000 [Reference]	0.21 (-0.5,0.1)	0.01 (-0.3,0.3)	
HDL-C (mmol/L)[1]				
Baseline	1.5 (1.3, 1.6)	1.4 (1.2, 1.5)	1.5 (1.3, 1.6)	
Follow-up	1.5 (1.3, 1.7)	1.4 (1.2, 1.6)	1.6 (1.4, 1.8)	
Change	0.0 (-0.1, 0.2)	0.1 (-0.1, 0.2)	0.2 (0.0, 0.3)	0.59
Treatment effect	0.000 [Reference]	0.02 (-0.1, 0.1)	0.07 (-0.2, 0.0)	
TC:HDL-C				
Baseline	3.3 (3.0, 3.7)	3.4 (3.0, 3.8)	3.3 (2.9, 3.7)	
Follow-up	3.2 (2.9, 3.6)	3.3 (3.0, 3.7)	3.1 (2.7, 3.4)	
Change	-0.1 (-0.3, 0.1)	-0.1 (-0.3, 0.1)	-0.2 (-0.4, 0.0)	0.96
Treatment effect	0.000 [Reference]	0.08 (-0.3, 0.2)	-0.12 (-0.4, 0.1)	
TAG (mmol/L)[1]				
Baseline	0.8 (0.7, 1.0)	0.8 (0.6, 1.0)	0.9 (0.7, 1.1)	
Follow-up	0.7 (0.6, 0.8)	0.7 (0.6, 0.9)	0.7 (0.6, 0.8)	
Change	-0.1 (-0.3, 0.1)	-0.1 (-0.3, 0.0)	-0.3 (-0.4, -0.1)	0.35
Treatment effect	0.000 [Reference]	0.04 (-0.2, 0.2)	-0.10 (-0.3, 0.1)	
NEFA (mmol/L)				
Baseline	0.3 (0.2, 0.4)	0.4 (0.3, 0.5)	0.4 (0.3, 0.5)	
Follow-up	0.4 (0.3, 0.4)	0.3 (0.2, 0.4)	0.3 (0.2, 0.4)	
Change	0.06 (-0.03, 0.15)	-0.09 (-0.18, 0.00)	-0.09 (-0.18, 0.00)	0.05
Treatment effect	0.000 [Reference]	-0.10 (-0.2, 0.0)	-0.10 (-0.2, 0.0)	

Measurements made at baseline after a 2-week run-in with placebo supplements and after 6 weeks with the placebo, DHA or EPA supplements at daily doses of 3 g. Association was tested by ANCOVA with baseline values as covariate. *P* values are adjusted for age, BMI and ethnicity. Values are means (95% CI). [1]Geometric mean.

7.9.5 Serum adiponectin concentration

Table 7.7 shows that there was a tendency for adiponectin concentrations to rise following placebo treatment but it remained unchanged on DHA or EPA treatments. There were no significant differences between EPA and DHA when compared with placebo.

Table 7. 7 Serum adiponectin concentration at baseline and after 6 weeks of supplementation with the placebo, DHA or EPA

Phenotype	Treatment group			
	Olive oil (*n* = 16) (Mean, 95% CI)	**DHA (*n* = 16)** (Mean, 95% CI)	**EPA (*n* = 16)** (Mean, 95% CI)	*P* value
Adiponectin (μg/L)[1]				
Baseline	6.1 (4.7, 8.0)	6.7 (5.1, 8.8)	6.3 (4.8, 8.2)	
Follow-up	7.4 (5.6, 9.9)	6.7 (5.0, 8.9)	6.3 (4.8, 8.4)	
Change	1.4 (0.6, 0.2)	0.0 (0.6, -1.2)	0.7 (0.6, -0.6)	0.08
Treatment effect	0.000 [Reference]	-0.19 (-0.4, 0.0)	-0.18 (-0.4, 0.0)	

Measurements made at baseline after a 2-week run-in with placebo supplements and after 6 weeks with the placebo, DHA or EPA supplements at daily doses of 3 g. Association was tested by ANCOVA with baseline values as covariate. *P* values are adjusted for age, BMI and ethnicity Values are mean (95% CI). [1]Geometric mean.

7.9.6 Insulin homeostatic variables

The results for plasma glucose, insulin concentrations and HOMA-IR are shown in **Table 7.8.** There was no evidence of any changes in plasma glucose but fasting insulin values tended to be greater on follow-up on all treatments. There were no differences between treatments in insulin or in HOMA, which an index of insulin sensitivity.

Table 7. 8 Plasma glucose, insulin and HOMA-IR at baseline and after 6 weeks of supplementation with the placebo, DHA or EPA

Phenotype	Treatment group			
	Olive oil (*n* = 16) (Mean, 95% CI)	**DHA (*n* = 16)** (Mean, 95% CI)	**EPA (*n* = 16)** (Mean, 95% CI)	*P* value
Fasting glucose (mmol/L)				
Baseline	5.0 (4.8, 5.1)	5.3 (5.1, 5.4)	5.2 (5.0, 5.4)	
Follow-up	5.1 (4.8, 5.4)	5.2 (5.0, 5.5)	5.2 (4.9, 5.5)	
Change	0.1 (-0.2, 0.4)	0.0 (-0.3, 0.3)	0.0 (-0.3, 0.2)	0.95
Treatment effect	0.000 [Reference]	-0.04 (-0.4, 0.4)	-0.08 (-0.5, 0.3)	
Fasting insulin (mU/L)[1]				
Baseline	5.4 (4.2, 7.0)	6.4 (5.0, 8.2)	5.6 (4.3, 7.2)	
Follow-up	6.7 (5.2, 8.7)	6.7 (5.1, 8.7)	6.1 (4.7, 7.9)	
Change	1.2 (1.0, 1.6)	1.0 (0.8, 1.3)	1.1 (0.9, 1.4)	0.72
Treatment effect	0.000 [Reference]	-0.11 (-0.4, 0.2)	-0.12 (-0.4, 0.2)	
HOMA-IR [1]				
Baseline	1.2 (0.9, 1.6)	1.5 (1.1, 2.0)	1.3 (1.0, 1.6)	
Follow-up	1.5 (1.2, 2.0)	1.5 (1.1, 2.2)	1.4 (1.0, 1.8)	
Change	0.2 (0.0, 0.5)	0.0 (-0.2, 0.2)	0.1 (-0.2, 0.4)	0.68
Treatment effect	0.000 [Reference]	-0.12 (-0.4, 0.2)	-0.14 (-0.5, 0.2)	

Measurements made at baseline after a 2-week run-in with placebo supplements and after 6 weeks with the placebo, DHA or EPA supplements at daily doses of 3 g. Association was tested by ANCOVA with baseline values as covariate. *P* values are adjusted for age, BMI and ethnicity Values are mean (95% CI). [1]Geometric mean

7.10 Discussion

The present study was conducted in men rather than women in order to avoid confounding effects of changes in the menstrual cycle.

7.10.1 Adiponectin and insulin sensitivity

The primary outcome of the study was to test whether relatively high intakes of EPA or DHA influenced serum adiponectin concentrations. The amounts provided by the supplements would be equivalent to amounts provided by large servings of oily fish. Compliance to the dietary supplements appeared to be good as indicated by capsules counts. There also were increases in the proportions of EPA and DHA in erythrocyte lipids. However, the increase in DHA in erythrocytes, however, was not possibly as great as reported in some previous studies and the expected fall in serum TAG was not observed. For example, Sanders et al. (2006) reported that a daily intake of 1.5g DHA for 6 weeks increased erythrocyte DHA from 6.1% to 7.6%, whereas in the present study, which provided 3g DHA, the increase in DHA was 6.0 to 7.7 %. The MARINA study (Sanders et al, 2011) reported that EPA and DHA increased by 1.3% to 3.6% and 6.5% to 8.3% respectively after 6 months supplementation with 1.8g of a mixture of EPA and DHA (with a ratio of EPA:DHA of 1.51). The increases in EPA and DHA in the present study were from 1.1% to 4.2% and 6.0 to 7.7% respectively.

The present study was unable to demonstrate any change in adiponectin following EPA or DHA compared with control. Indeed, the tendency was a reduction rather than an increase in adiponectin compared with placebo. The present study measured total adiponectin, rather than HMW adiponectin, which has been shown to be at its most active when mediating the adiponectin metabolic effect (Kobayashi et al, 2004).

We were unable to find any significant changes in fasting insulin, glucose or HOMA-IR after EPA or DHA supplementation. These findings are in line with the findings of the Hartweg meta-analysis, where 9 weeks of 3.5 g/d n-3 PUFA had no effect on fasting insulin or glycaemic control (Hartweg et al, 2008). However, in hyperlipidaemic men, EPA and DHA taken individually increased fasting insulin but only EPA increased fasting glucose (Mori et al, 2000). A limitation of the present study is that the participants were young and healthy and unlikely to have compromised pancreatic beta-cell function.

7.10.2 Blood lipids

n-3 fatty acids regulate PPARs and SREBPs, which regulate the expression of lipid homeostasis genes (Deckelbaum et al, 2006; Yu et al, 2011) and increase the expression of the lipolytic genes (Yu et al, 2011). DHA, uniquely among PUFAs, controls SREBP-1 nuclear abundance through 26S proteasomal degradation, and suppression of SREBP-1, which in turn suppresses lipogenesis (Jump et al, 2008). Several studies have investigated the differential effect of EPA and DHA in blood lipids (Harris et al, 2008; Harris, 1989), which suggested that both EPA and DHA contribute to the TAG lowering effect. Using lipoprotein turnover studies with labelled substrate it has been demonstrated that the TAG lowering effect following EPA and DHA consumption is primarily mediated by a substantial decrease in hepatic TAG synthesis and VLDL TAG secretion (Harris et al, 1990; Sanders et al, 1985; Nestel et al, 1984) without any effect on catabolic rate. However, there are limited studies indicating increased clearance rates of VLDL (Bordin et al, 1998). DHA was found to lower TAG concentration in healthy subjects (Grimsgaard et al, 1997; Agren et al, 1996); in hypertensive diabetic patients (Woodman et al, 2002), and in dyslipidaemic subjects (Nestel et al, 2002; Mori et al, 2000; Kelley et al, 2007). However, Theobald et al. (2004) and Sanders et al. (2006) were unable to show significant TAG lowering effects of 0.7g and 1.5g DHA/d. The present study which provided doses of ~3g/d was unable to demonstrate any significant changes in blood lipids. However, basal fasting TAG was low and it is known that the plasma TAG lowering effect is more evident in individuals with raised plasma TAG. There was a tendency for Apo B concentrations to be slightly greater following DHA which is in agreement with previous studies using algal DHA (Theobald et al, 2004; Sanders et al, 2006; Kelley et al, 2007). A limitation of the present study in this respect is the small sample size for a parallel design.

7.11 Limitations and conclusion

This study was conducted in young healthy men and so the results cannot be extrapolated to women or to older men and women especially as adiponectin concentration increase with increase age.

Chapter 8

Final Discussion and Conclusions

8.1 Discussion

This thesis set out to test the hypothesis the type of fatty acids in the diet would influence adiponectin concentrations and this may be influenced by common polymorphism in *ADIPOQ*, *PPARG*, and *PPARA* genes. It was found that adiponectin was positively correlated with age and negatively with BMI. Adiponectin was significantly higher in White Europeans than in South Asians and Black Africans. The ethnic differences are consistent with other reports but do not appear to be due to difference in dietary fatty acid intakes between groups. Serum adiponectin concentrations were significantly higher in females than males. The effects of four SNPs (-11391 G/A, -10066 G/A, -7734 C/A and +276 G/T) in the *ADIPOQ* gene and adiponectin concentrations in RISCK White subjects were investigated. The selected SNPs were previously showed to have the strongest replicated associations with serum adiponectin (Kyriakou et al, 2008). There was a small effect of diet high in MUFA and -10066 G/A. After HM diet, GG subjects showed a 3.8% increase and GA+AA subjects a 2.6% decrease in serum adiponectin. The switch from SFA to MUFA could lead to increased expression of the *ADIPOQ* gene and serum adiponectin concentration through increased availability of PPARγ-activating ligands. The PPRE lies in a 1.3 kb linkage disequilibrium block (Heid et al, 2006). If the -10066A-allele was in LD with a variant in the PPRE reducing affinity for the receptor, this could account for higher serum adiponectin in response to MUFA in GG homozygotes and the lower concentration in A-allele carriers. This effect appeared to become exaggerated with increasing age. In -10066 GG homozygotes over 40 years of age, adiponectin concentration increased progressively after the HM diet and decreased after the LF diet. The difference in % change in serum adiponectin between GG subjects on HM and LF diets in the oldest 61-70 year age group achieved statistical significance. In -10066 A-allele carriers there was little change in serum adiponectin concentration compared to baseline with increasing age, after HM or LF diet. The major limitation is the small number of subjects; thus it is important in replicating the findings in a larger cohort of older subjects and robust evidence would be provided from repeated measures from prospective studies.

The reasons for this apparent increase in adiponectin with age are uncertain. The analyses for the current study were corrected for adiposity; therefore changes in adiposity with increasing age might not be the reason. Fetal programming and early

development may have an effect on adiponectin expression in later life and this may also explain some of the differences between ethnic groups (Bansal et al, 2011). It is possible that there are developmental effects on pre-adiposites in early life that affect the capacity to synthesis adiponectin in later life in response to adipose tissue accumulation.

In the RISCK cohort, substituting high saturated diet with either high MUFA diet or carbohydrate (low fat diet) has no effect on serum adiponectin concentrations. All three diets contained the same proportion of PUFA. The lack of effect of replacing MUFA with carbohydrate is in contrast to a report from the OMNIHEART investigators (Yeung et al, 2010). They reported that a MUFA diet maintained a higher level of both HMW and total adiponectin concentrations than either a carbohydrate rich low fat diet (HMW: +6.8%, $P = 0.02$; total: +4.5%, $P = 0.001$) or a protein rich diet (HMW: +8.4%, $P = 0.003$; total: +5.6%, $P < 0.001$) diets. The duration of the intervention in that study was only 6 weeks as opposed to six months in the RISCK study. However, the findings are broadly in agreement with the findings in participants with the -10066 GG genotype in RISCK. Therefore, we conducted a dietary intervention trial investigating the effect of LC PUFAs on adiponectin concentrations. The amounts provided by the supplements would be equivalent to amounts provided by large servings of oily fish. This had no effect on serum adiponectin concentrations. Therefore, it is unlikely that lower amounts of LC PUFA found in a typical diet (< 1g/d) would have any effect on adiponectin in adult life. A limitation of this study was that it was conducted in young healthy men and so the results cannot be extrapolated to women or to older men and women. Further research could involve measuring changes in serum adiponectin in the MARINA study according to genotype.

These findings suggest that age, gender, BMI and ethnicity are major determinant of adiponectin concentration. We reported a modest effect of diet and -10066 G/A, which exaggerated with increasing age. High intake of LC PUFAs has no effect of adiponectin concentrations in healthy males.

Cross sectional analysis confirms the association between P:S ratio and *PPARG* Pro12Ala as previously reported by Luan et al.(2001). An analysis of White subjects in the RISCK study, showed that at the lowest P:S intake, carriage of the less active PPAR-γ Ala12 isoform was associated with higher plasma TC and LDL-C. Significant

trends in the reduction of plasma TC and TAG in Ala12-allele carriers as the P:S ratio increased. Thioglitazone decreases the release of NEFA from adipose tissue, reducing TAG synthesis in the liver and decreasing VLDL synthesis, which in turn has an effect on LDL-C, but has a greater effect in raising HDL-C (Belfort et al, 2006). The PPARγ-Ala12 form has lower transactivational ability than the wild-type (Deeb et al, 1998). However a mechanistic link to PPARγ target gene activation, that might infer association of the less active PPARγ-Ala form with lower LDL-C concentration, has not been established. The decrease in TAG concentration in Ala12 carriers as the P:S ratio increase is consistent with Lindi's findings in 2003. The former study found significantly greater decrease in serum TAG concentration in Ala12 carriers than in Pro12 homozygotes in response to *n*-3 fatty acid supplementation, when the intake of SFA was below 10%, i.e. at high P:S intake. However, both studies are inconsistent with reduced lipoprotein lipase activity associated with a less-active PPARγ-Ala isoform.

In this study, we also showed that the significant interaction between P:S and *PPARG* Pro12Ala as a determinant of plasma lipids did not depend on decrease SFA intake. However, in the MARINA study no significant interaction between *PPARG* Pro12Ala and LC PUFA as determinate of plasma lipids concentrations was found. The difference between the participants in MARINA and RISCK study were mainly in BMI. PPARγ undoubtedly plays an important role in insulin sensitivity and influence *ADIPOQ* gene expression. However, the findings in this thesis do not provide evidence for an effect of LC PUFAs or saturated fatty acids on PPARγ. Adiponectin, insulin and glucose concentrations have yet to be analysed in the MARINA study. This will enable us to test the interaction between *PPARG* Pro12Ala and LC PUFA as determent of HOMA-IR and adiponectin concentrations.

The role of PPARα is primarily to activate the peroxysomal beta-oxidation of fatty acids in the liver, particularly $C_{20\text{-}22}$ unsaturated fatty acids and branched chain fatty acids so that they can be reduced to sufficient size to form carnitine esters that can enter the mitochondrial for oxidation. This redirection of fatty acids from TAG synthesis to oxidation explains the reduction in VLDL TAG synthesis and secretion that occurs following the feeding of n-3 LCP, but does not occur with linolenic acid. PPARα may also have an additional role as activators of LPL activation. There is little evidence for an increase in fractional catabolic rate of VLDL from human feeding studies with

increased intakes of n-3 LCP, which would suggest that the main effect is on synthetic rate. In the MARINA study, we found a significant interaction n-3 PUFAs supplementation and *PPARA* Leu162Val as determinant of plasma TAG concentration. n-3 PUFAs supplementation lowered plasma TAG concentration significantly in *PPARA* Leu162 homozygotes, with significant increase between the highest dose compared to placebo but not in Val162-allele carriers. The *LPL* gene is responsive to PPARα activation (Schoonjans et al, 1996). Transcription of *LPL* was found to be higher in Leu162- than Val162-constructs containing the *LPL* PPRE, after *n*-3 fatty acid transactivation (Rudkowska et al, 2009). This is compatible with the decreased TAG concentration we found in subjects homozygous for the more transcriptionally active Leu162 allele.

A novel finding of the thesis is the potential interaction between *PPARG* Pro12Ala and *PPARA* Leu162 Val and dietary intake of fat as determinant of plasma lipid. We reported a significant interaction between the two genotypes as determinants of plasma LDL-C concentration and sdLDL as a proportion of LDL after adjustment for change in BMI, age and gender. Carriage of both variant alleles (*PPARA* Val162 and *PPARG* Ala12) was associated with a greater reduction in LDL-cholesterol and proportion as sdLDL after HM diet than after LF diet. PPARγ-Ala12 and PPARα-Val162 forms have lower transactivational ability than the wild-types (Deeb et al, 1998; Rudkowska et al, 2009).

At baseline, after a 4-week run-in on the HS diet, we found no significant independent association between *PPARA* Leu162Val or *PPARG* Pro12Ala and proportion of sdLDL. Similarly, Bouchard-Mercier et al. (2011) found no significant change in LDL-PPD in *PPARG* Pro12 homozygotes or Ala12-allaele carriers after high SFA intake, but a significant increase in LDL-PPD in Ala12 carriers after high intake of PUFA. In contrast to our findings, they found that high SFA intake associated with larger LDL particle size in *PPARA* Leu162 homozygotes, but with a higher proportion of sdLDL in Val162 carriers. We found no significant change in the proportion of sdLDL in carriers of *PPARG* Ala12 or *PPARA* Val162 on switching from the HS diet at baseline to the HM or LF diets, but a significant reduction in the proportion of sdLDL in carriers of both *PPARA* Val162 and *PPARG* Ala12 alleles after the HM diet. This cannot be explained by reduced activity of both variants, but would be expected to lead to a higher proportion of sdLDL. The concentration of sdLDL is more dependent on VLDL TAG

concentrations because TAG is transferred by cholesterol ester transfer protein from VLDL to LDL, which in turn can be converted to sdLDL by hepatic lipase (Sanders & Emery, 2003). A high carbohydrate low fat diet would be expected to increase VLDL-TAG. However, in the RISCK study there were no differences in fasting TAG, possibly because subjects received a standardised low fat meal on the day preceding blood collection.

A major limitation of this study that only 7 subjects were carriers of both *PPARA* 162Val and in *PPARG* Ala12 variants and these showed a significant reduction in plasma LDL-C and its proportion as sdLDL after the HM diet, as compared to the LF diet. In the MARINA study only one subject proved to be the carrier of the minor variants of both SNPs. A larger sample is required to confirm this association, which may be achieved by recruiting subjects based on the presence of both variants and randomising them to either the HM or LF diet.

8.2 Concluding remarks, limitation and implications for further research

The strength of the RISCK and MARINA studies lies in their design as randomised tightly controlled feeding trials with high adherence and retention rates and diets with practical relevance to the general population. Limitations to these SNP association studies include relatively small sample sizes and reduced power after randomisation to dietary treatments, in comparison with measurements at baseline. In the RISCK and MARINA studies, we focused our genetic analysis on White subjects. Replicating the findings in other ethnic group populations is required. Replication of all our findings in other studies with maximal correspondence in ethnic origin, age, and gender would be required to minimise the risk of false positive or negative gene-diet associations. Substantiated effects of common SNPs in modifying the outcome of dietary intervention studies in larger samples should help in the identification of individuals at risk of complex disease who would benefit from personalised dietary recommendations

The major problem with genome wide association studies is that very high levels of statistical significance are required to allow for multiple comparisons. This means that real diet x genetic interactions may be missed. A promising approach would be to using a global risk scoring system to reduce the penalties incurred by multiple comparison testing For example, Walker et al. (2011) have used such an approach in examine risk

allele for serum cholesterol and insulin resistance. This study set out to examine whether variations in dietary fat influenced adiponectin concentrations. The findings presented in this thesis suggest no effect of EPA or DHA on adiponectin but a modest of MUFA compared to low fat, which appeared to be specific to a specific genotype. Large effects of age, gender, obesity and ethnicity were found that require further exploration.

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Appendices

Appendix 2.1 MARINA study experimental oils

The experimental oils were supplied by Croda Europe (Hull, UK) and encapsulated by Powerhealth (Pocklington, UK). Blends of the test fat with 0.1 wt % peppermint oil to disguise the fish taste of the EPA and DHA were supplied. The placebo contained refined olive oil plus 0.1% peppermint oil. The EPA and DHA blends consisted of blends of an EPA concentrate (TG7010, code SF06396, batch 213000) and a DHA concentrate (DHA700TG, code SF06405, batch 213003) and refined olive oil (batch 10813). These were blended to provide to provide to 1.8g, 0.9 and 0.46g EPA+DHA (with an EPA/DHA ratio of 1.51). The capsules (1g fill) were analyzed by the quality control laboratory at Croda Europe.

Appendix 2.2 Analysis of serum adiponectin:

AutoDELFIA -based-time-resolved-fluorescence based assay is a method used for detecting human total serum adiponectin (low, middle, and high molecular weight). In this method surface of wells in microtite plates is coated with mouse antibody to capture a target antigen (adiponectin) which are then further bound by europium (Eu) labeled antibodies that therefore, detect the presence of adiponectin .

Materials
PerkinElmer Victor 2 multilabel counter
PerkinElmer AutoDELFIA 1235 automatic immunoassay system
PerkinElmer DELFIA platewasher 1296-026
PerkinElmer Plateshaker 1296-003
Perkin Elmer Assay Buffer (1244-111)
Perkin Elmer Enhancement Solution (1244-105)
Perkin Elmer wash concentrate (1244-114)
Perkin Elmer Tracer Stability Buffer (CR84-100)
96 well high binding fluoroNunc solid flat-bottomed microplates

Note: All reagents and serum samples were thawed, mixed and brought to room temperature before analysis.

Figure 2.1 AutoDELFIA (Perkin Elmer) is an automated fluorescent immunoassay system that is specifically designed to perform all sample and reagent handling

Into each well of the microtitre plate, mouse antibody was absorbed. Plates were washed this is followed by adding 100 µl serum Adiponectin to the wells in duplicate, with 1/10000 dilution in Perkin Elmer DELFIA assay buffer. Adiponectin present is captured by antibody and unbound protein is washed away after the first incubation period. Into each well 100 µl biotinlylated detection antibody added to each well. Then plates were further washed and 100 µl streptavidin Eu conjugate added (europium labelled antibody) to each well and were incubated again. Plated were then washed 6

times and 200 µl enhancement solution was added to each well. The plated were then incubated for 5 minutes before reading via a multi analyte detection system, set up for the detection for free Eu that detects the adiponectin.

Appendix 2.3 Insulin resistance (Si)

Glucose in the form of a 50% solution (0.3 g/kg) and regular human insulin (0.03 units/kg) were injected through an intravenous cannula at 0 and 20 minutes respectively. Blood was collected at –10, -5, 0, 2, 4, 8, 19, 22, 30, 40, 50, 70, 100 and 180 min for measurement of plasma glucose and insulin concentrations. Si was calculated by mathematical modelling methods using the MINMOD Millennium programme (Version 6.02). From the model, coefficients were obtained which may be interpreted as the acceleration of glucose disposal due to an incremental change in plasma glucose (glucose effectiveness, Sg), and the contribution attributable to an incremental rise in plasma insulin (Si). Sg and Si can therefore be taken as indices of insulin-independent and insulin-assisted glucose disposal respectively. In addition, the area under the plasma insulin curve in the early stages of the test was computed and used as an indicator of the degree of endogenous insulin secretion in response to the glucose challenge (AIR_G). This term was combined with Si to give the disposition index (DI), which is a quantitative measure of the amount of glucose disposed, by the insulin-dependent pathway during the test.

Appendix 2.4 Anthropometry

Weight was measured after an overnight fast to the nearest 0.1 kg using digital scales. Measurements were made with participants wearing light clothing and without shoes. Height was measured to the nearest 0.1 cm using a wall-mounted stadiometer. Waist circumference was measured to the nearest 0.1 cm in the standing position with arms to the side, shoulders-width apart and weight equally distributed on each leg. The measurement was standardised as the mid-point between the lower rib margin and the iliac crest and, where possible, was taken on participants with their waist uncovered or else without heavy outer garments. Participants were asked to breathe normally and the reading was taken at the end of a normal exhalation. Body composition was assessed using a Tanita BC-418 segmental body composition analyser (Tanita Corporation of America, Inc., Illinois USA).

Appendix 2.5 Seated blood pressure

Blood pressure and heart rate was taken using an OMRON 705CPII or equivalent auto upper arm blood pressure monitor. These measures were taken while the participant is seated and after 10 minutes of rest. When taking a blood pressure measurement the volunteer was calm and relaxed, sitting quietly with their legs uncrossed and feet flat on the floor the arm that the measurement was being taken from needs was supported. A cuff of appropriate size was selected and placed around upper arm wrapped snugly around the arm with space for a finger between volunteer and cuff. The cuff was appropriately aligned with the brachial artery following the instructions contained in the equipment manual. Measures of systolic and diastolic blood pressure and heart rate were made. The blood pressure measurements were repeated at least three times at two minute intervals. The value for the first reading was discarded and the mean for the two following readings is taken according to the British Hypertension Society Guidelines.

Appendix 3.1: Table 3.1 Characteristics of RISCK study subjects at baseline

Phenotype	All (n= 448)	Women (n= 184)	Men (n=264)	*P*
Ethnicity[1]				
South Asia	44(9.8)	19(10.3)	25(9.4)	
Black African	38(8.5)	10(5.4)	28(10.6)	
White European	366(81.7)	155(84.3)	211(80)	
Age (y)	52.5 ± 9.9	51.8 ± 9.5	53.6 ± 10.2	0.05
BMI (kg/m^2)	28.8 ± 4.7	28.9 ± 5.2	28.5 ± 3.9	0.43
Fasting insulin (pmol/L)[2]	60.4 ± 52.1	58.2 ± 58.7	63.4 ± 40.8	0.1
Fasting glucose (mmol/L)	5.7 ± 0.8	5.5 ± 0.6	5.9 ± 0.9	<0.001
Insulin sensitivity IVGTT)((mU/L)-1 min-1)[2]	2.7 ± 2.7	2.9 ± 3.1	2.4 ± 1.9	0.001
HOMA2-IR[2]	1.3 ± 0.7	1.3 ± 0.7	1.3 ± 0.8	0.54
Adiponectin (μg/mL)[2]	9.6+5.9	11.1+6.3	7.7+4.1	<0.001
TAG (mmol/L)[2]	1.4+0.7	1.3+0.6	1.5+0.8	<0.001
TC (mmol/L)	5.6+1.0	5.6+1.0	5.6+1.0	0.56
HDL-C (mmol/L)	1.4+0.3	1.5+0.3	1.3+0.3	<0.001
ApoA1 (g/L)	1.2+0.2	1.2+0.2	1.2+0.2	0.003
LDL-C (mmol/L)	3.5+0.8	3.5+0.8	3.6+0.8	0.16
Apo B (g/L)	1.0+0.3	1.0+0.3	1.0+0.3	0.11
Systolic BP (mm Hg)	129.6+15.9	125.7+15.2	135.2+15.3	<0.001
Diastolic BP (mm Hg)	79.6+9.5	77.3+9.1	82.9+9.1	<0.001

Data is presented for subjects which DNA samples were available (*n* = 448). Mean ± SD, or [2]geometric mean ± SD values for insulin, Si, HOMA2-IR, adiponectin, TAG and HDL-C are shown. All variables were measured at baseline after 4-wk run-in on reference HS diet. [1]Self-reported ethnicity. Significance of differences between women and men was determined by T-test

Appendix 3.2 Genotyping by Pyrosequencing

Pyrograms for the three *ADIPOQ* -7734 C/A, 11391 G/A and. +276 G/T genotypes are shown below

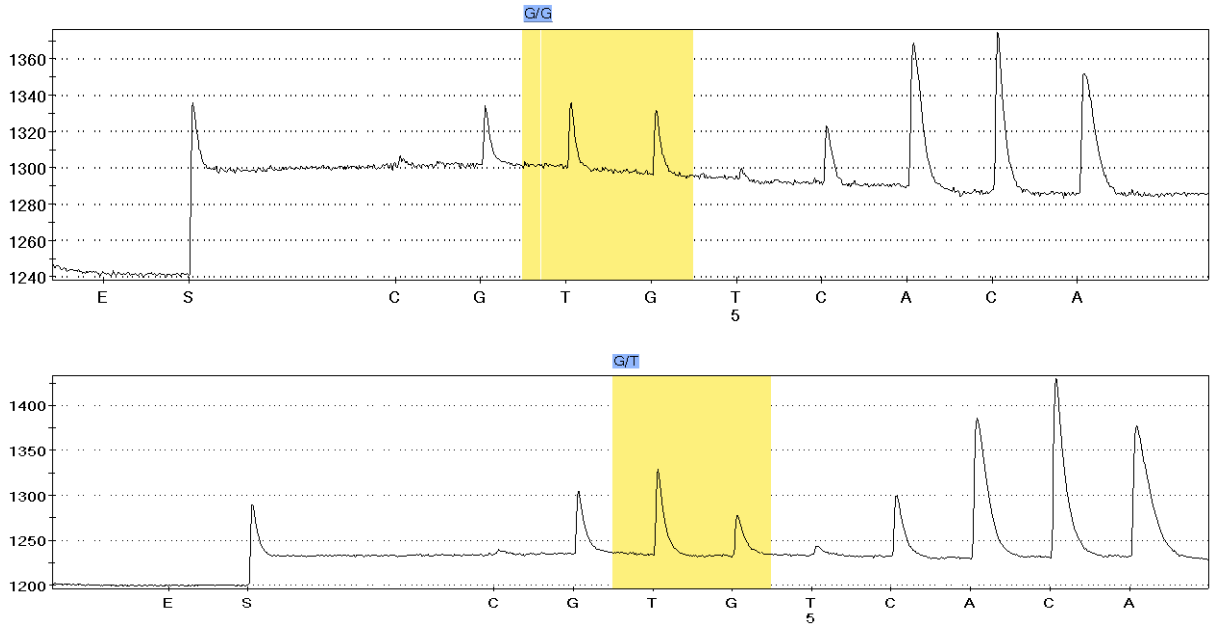


Figure 3.1 Pyrograms for *ADIPOQ* -7734 C/A genotypes Yellow blocks indicate the peaks for the analysed SNP

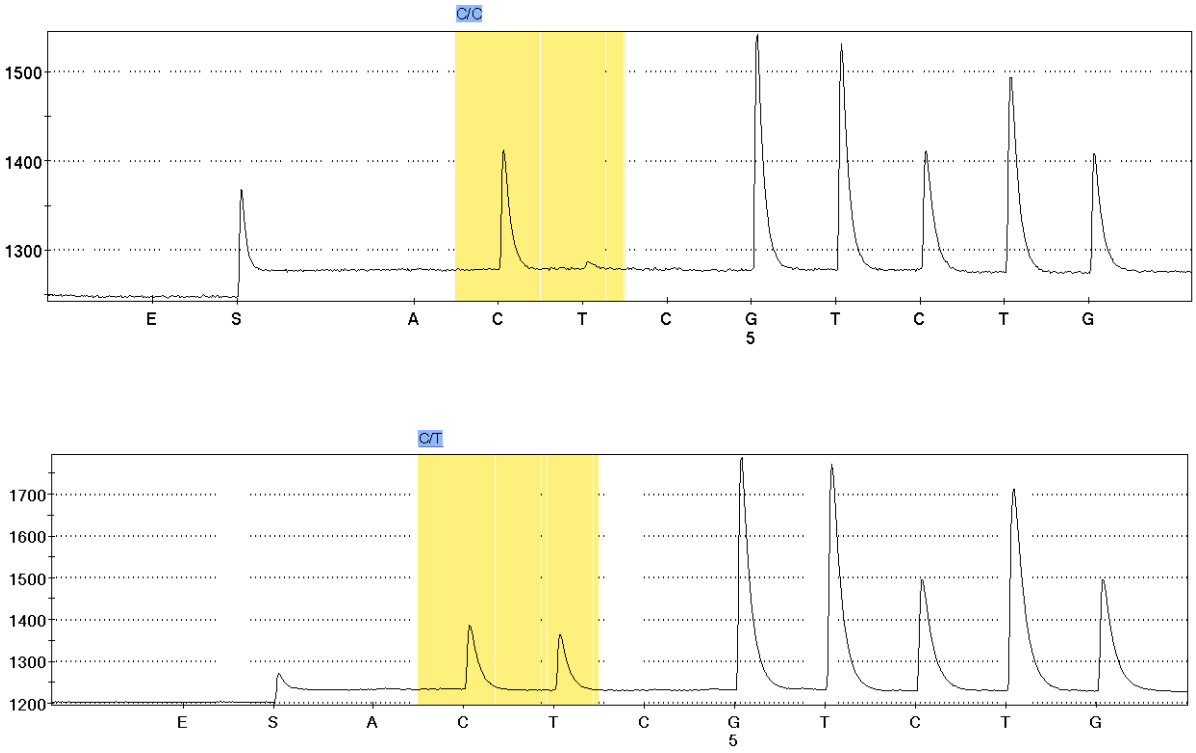


Figure 3.2 Pyrograms for *ADIPOQ* 11391 G/A genotypes Yellow blocks indicate the peaks for the analysed SNP

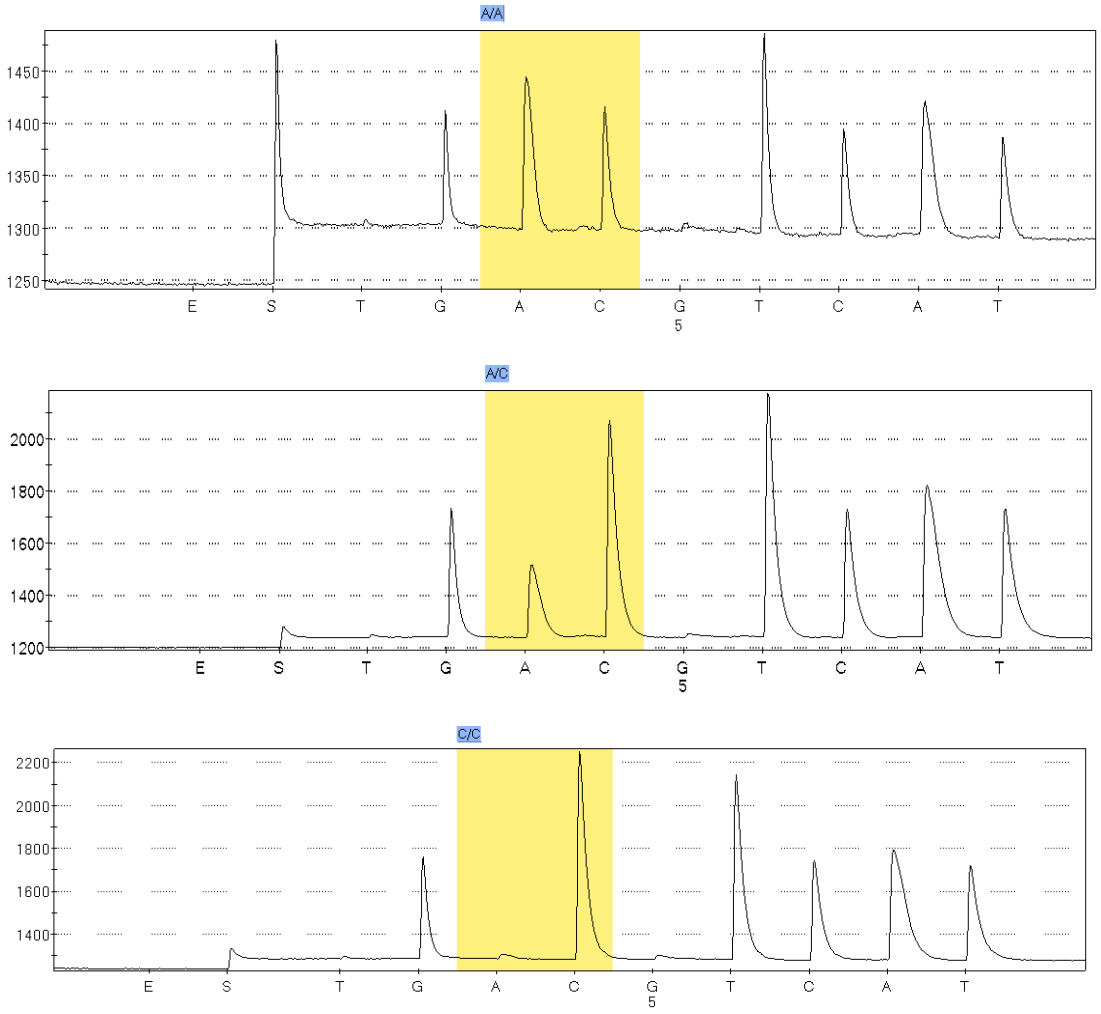


Figure 3.3 Pyrograms for *ADIPOQ* -276 G/T genotypes Yellow blocks indicate the peaks for the analyse

Appendix 5.1: Table 5.1 Effect of Interaction between fat intake and *PPARA* Leu162Val genotypes in determining changes in obesity measures, insulin sensitivity measures and plasma lipids and adiponectin in White subjects

	HS		HM		LF		
Phenotype	LL (*n* = 45)	LV (*n* = 7)	LL (*n* = 128)	LV (*n* = 22)	LL (*n* = 137)	LV (*n* = 17)	*p*
	Change (95%CI)	Change (95%CI)	Change (95%CI)	Change (95%CI)	Change (95%CI)	Change (95%CI)	diet x gene
BMI (kg/m2)[1]	0.1 (-0.2,0.4)	0.5 (0.0,1.1)	0.0 (-0.2,0.1)	-0.1 (-0.5,0.3)	-0.4 (-0.6,-0.2)	-0.3 (-1.0,0.4)	0.45
Waist circumference (cm)[1]	-0.6 (-1.9,0.7)	-0.3 (-3.6,3.1)	0.1 (-0.7,0.8)	-1.1 (-3.5,1.4)	-1.4 (-2.2,-0.6)	-0.4 (-2.7,1.9)	0.33
Body fat %[1]	0.3 (-0.3,1.0)	1.2 (-0.4,2.8)	-0.3 (-0.7,0.0)	0.2 (-0.9,1.2)	-0.5 (-0.9,-0.1)	-0.1 (-1.4,1.3)	0.92
Insulin sensitivity IVGTT)((mU/L)-1 min-1) [2]	-0.02 (-0.1,0.1)	-0.1 (-0.36,0.21)	-0.04 (-0.1,0.1)	0.0 (-0.18,0.21)	0.02 (-0.1,0.1)	-0.1 (-0.31,0.12)	0.58
Fasting insulin (pmol/L)	-2.9 (-3.2,-2.7)	-3.2 (-4.1,-2.3)	-3.4 (-3.6,-3.1)	-2.9 (-3.4,-2.4)	-2.9 (-3.1,-2.8)	-3.2 (-3.7,-2.6)	0.11
Fasting glucose (mmol/L) [2]	-0.05 (-0.16,0.06)	0.04 (-0.43,0.51)	-0.10 (-0.16,-0.03)	-0.30 (-0.95,0.36)	-0.16 (-0.27,-0.05)	-0.21 (-0.45,0.02)	0.58
HOMA2-IR[2]	0.04 (-0.07,0.15)	0.13 (-0.22,0.49)	0.05 (-0.03,0.13)	-0.01 (-0.22,0.19)	0.00 (-0.09,0.08)	0.03 (-0.22,0.29)	0.68
NEFA (µmol/L)	-61.3 (-130.6,8.0)	-109.2 (-304.4,86.0)	-34.3 (-77.3,8.7)	-10.4 (-167.6,146.7)	3.4 (-36.8,43.6)	3.6 (-91.6,98.9)	0.84
Total cholesterol (mmol/L)	-0.05 (-0.22,0.12)	0.09 (-0.24,0.41)	-0.37 (-0.47,-0.27)	-0.38 (-0.69,-0.07)	-0.40 (-0.49,-0.31)	-0.42 (-0.69,-0.15)	0.92
TAG (mmol/L) [2]	0.00 (-0.10,0.09)	0.09 (-0.30,0.49)	-0.04 (-0.09,0.01)	0.00 (-0.17,0.18)	-0.01 (-0.05,0.03)	0.01 (-0.15,0.17)	0.94
HDL Cholesterol (mmol/L) [2]	-0.05 (-0.12,0.02)	-0.07 (-0.30,0.15)	-0.04 (-0.07,-0.01)	-0.07 (-0.15,0.01)	-0.11 (-0.14,-0.08)	-0.10 (-0.19,-0.01)	0.85
Apolipoprotein A1 (mg/dL)	-0.02 (-0.07,0.02)	0.03 (-0.13,0.20)	-0.01 (-0.04,0.01)	-0.05 (-0.13,0.03)	-0.07 (-0.10,-0.04)	-0.08 (-0.21,0.04)	0.76
LDL Cholesterol (mmol/L)	0.01 (-0.14,0.16)	0.00 (-0.34,0.34)	-0.29 (-0.37,-0.22)	-0.35 (-0.63,-0.06)	-0.28 (-0.36,-0.20)	-0.31 (-0.49,-0.12)	0.98
Apolipoprotein B (mg/dL)	0.03 (-0.02,0.08)	0.05 (-0.14,0.24)	-0.06 (-0.09,-0.03)	-0.11 (-0.19,-0.03)	-0.07 (-0.11,-0.04)	-0.08 (-0.18,0.02)	0.75
Proportion of sdLDL (%)	1.34 (-1.40,4.08)	8.51 (-5.69,22.70)	2.24 (0.36,4.12)	-0.88 (-5.98,4.22)	1.72 (0.20,3.23)	3.76 (0.13,7.39)	0.1
Adiponectin (µg/mL)	0.04 (0.02,0.09)	-0.04(-0.21,0.13)	-0.02 (-0.04,0.02)	0.08(0.03,0.16)	-0.01(-0.04,0.02)	0.01 (-0.09,0.11)	0.1

Values are mean change (95% CI) or [2]GM (95% CI. All data and *P*-values for ANCOVA adjusted for age, gender and BMI, based on subjects for whom *PPARA* Leu162Val genotype data was available (*n* = 356). LL represents subjects homozygous for the *PPARA* Leu162 allele and LV carriers of the Val162 allele.[1]P-values adjusted for gender and age.

Appendix 5.2 Table 5.2 Effect of Interaction between fat intake *PPARG* Pro12Ala genotypes and changes in obesity measures, insulin sensitivity measures and plasma lipids and adiponectin concentrations in White subjects

	HS		HM		LF		
Phenotype	PP (*n* = 47)	PA+AA (n = 7)	PP (*n* = 109)	PA + AA (*n* = 20)	PP (*n* = 109)	PA + AA (*n* = 37)	*p*
	Change (95%CI)	Change (95%CI)	Change (95%CI)	Change (95%CI)	Change (95%CI)	Change (95%CI)	diet x gene
BMI (kg/m2)[1]	0.1 (-0.1,0.4)	0.3 (-0.3,0.9)	-0.1 (-0.2,0.1)	-0.1 (-0.6,0.3)	-0.5 (-0.7,-0.3)	-0.2 (-0.5,0.2)	0.42
Waist circumference (cm)[1]	-0.5 (-1.7,0.7)	-1.5 (-6.1,3.1)	-0.3 (-1.1,0.6)	-0.2 (-2.3,1.9)	-1.5 (-2.4,-0.6)	-1.2 (-2.6,0.1)	0.81
Body fat %[1]	0.5 (-0.2,1.1)	1.0 (-0.5,2.6)	-0.2 (-0.6,0.2)	0.0 (-0.8,0.8)	-0.4 (-0.9,0.0)	-0.2 (-1.1,0.6)	0.94
Insulin sensitivity IVGTT)((mU/L)-1 min-1) [2]	-0.08 (-0.2,0.1)	0.0 (-0.31,0.38)	-0.05 (-0.2,0.1)	-0.1 (-0.26,0.06)	0.02 (-0.1,0.1)	0.1 (-0.09,0.21)	0.75
Fasting insulin (pmol/L)[2]	-3.0 (-3.3,-2.7)	-2.9 (-3.5,-2.3)	-3.3 (-3.6,-3.0)	-3.3 (-3.6,-3.0)	-3.1 (-3.3,-2.9)	-2.8 (-3.2,-2.4)	0.81
Fasting glucose (mmol/L)	-0.05 (-0.15,0.05)	-0.05 (-0.38,0.27)	-0.13 (-0.28,0.01)	-0.08 (-0.23,0.08)	-0.12 (-0.19,-0.06)	-0.32 (-0.70,0.07)	0.41
HOMA2-IR[2]	0.01 (-0.10,0.12)	0.01 (-0.31,0.34)	0.02 (-0.06,0.10)	0.08 (-0.08,0.24)	0.04 (-0.05,0.13)	-0.06 (-0.23,0.11)	0.62
NEFA (μmol/L)	-68.8 (-138.6,1.0)	-95.7 (-301.3,110.0)	-8.1 (-52.9,36.6)	-4.6 (-157.3,148.0)	-25.4 (-72.9,22.1)	85.5 (18.1,153.0)	0.24
Total cholesterol (mmol/L)	-0.04 (-0.20,0.12)	-0.04 (-0.65,0.57)	-0.33 (-0.44,-0.21)	-0.38 (-0.56,-0.21)	-0.38 (-0.48,-0.29)	-0.46 (-0.66,-0.25)	0.92
Triglycerides (mmol/L) [2]	0.04 (-0.05,0.12)	-0.07 (-0.45,0.31)	-0.01 (-0.07,0.05)	0.00 (-0.15,0.15)	-0.01 (-0.06,0.04)	-0.06 (-0.15,0.04)	0.55
HDL Cholesterol (mmol/L) [2]	-0.04 (-0.11,0.02)	-0.03 (-0.14,0.09)	-0.05 (-0.08,-0.02)	-0.04 (-0.11,0.04)	-0.10 (-0.14,-0.07)	-0.14 (-0.19,-0.08)	0.64
Apolipoprotein A1 (g/L)	0.01 (-0.04,0.06)	-0.07 (-0.20,0.05)	-0.01 (-0.04,0.01)	-0.04 (-0.11,0.04)	-0.05 (-0.09,-0.02)	-0.10 (-0.16,-0.04)	0.56
LDL Cholesterol (mmol/L)	-0.03 (-0.18,0.13)	0.09 (-0.26,0.43)	-0.29 (-0.38,-0.20)	-0.32 (-0.54,-0.09)	-0.27 (-0.35,-0.19)	-0.27 (-0.44,-0.10)	0.8
Proportion of sdLDL (%)	2.38 (-0.80,5.56)	-1.07 (-5.28,3.15)	2.66 (0.72,4.59)	-2.30 (-7.10,2.49)	1.72 (0.39,3.06)	1.78 (-1.68,5.25)	0.31
Apolipoprotein B (g/L)	0.04 (-0.01,0.09)	-0.04 (-0.22,0.14)	-0.06 (-0.09,-0.03)	-0.06 (-0.13,0.01)	-0.07 (-0.10,-0.03)	-0.08 (-0.16,0.00)	0.94
Adiponectin (μg/mL)	0.02 (-0.03,0.08)	-0.08 (-0.15,-0.01)	-0.01 (-0.04,0.02)	0.03 (-0.03,0.09)	0.00 (-0.02,0.03)	-0.03 (-0.09,0.03)	0.16

Values are mean change (95% CI) or [2]GM (95% CI). All data and *P*-values for ANCOVA adjusted for age, gender and BMI, based on subjects for whom *PPARG* Pro12Ala genotype data was available (*n* = 329). PP represents subjects homozygous for the *PPARG* Pro12 allele and PA + AA carriers of the Ala12 allele.[1]P-values adjusted for gender and age.

Appendix 5.3: Table 5.3 Effect of Interaction between fat intake and *PPARA* Leu162Val genotypes in determining obesity measures, insulin sensitivity measures and plasma lipids and adiponectin in White subjects

	HS		HM		LF	
Phenotype	LL (*n* = 45)	LV (*n* = 7)	LL (*n* = 128)	LV (*n* = 22)	LL (*n* = 137)	LV (*n* = 17)
	Follow-up (95%CI)	Follow-up (95%CI)	Follow-up (95%CI)	Follow-up (95%CI)	Follow-up (95%CI)	Follow-up (95%CI)
BMI (kg/m2)	28.7 (28.4,29.0)	30.5 (29.7,31.4)	28.6 (28.4,28.7)	29.9 (29.4,30.4)	28.2 (28.0,28.3)	29.7 (29.2,30.2)
Waist circumference (cm)	97.6 (96.4,98.9)	99.2 (95.5,102.9)	98.3 (97.6,99.0)	98.4 (96.3,100.6)	96.8 (96.1,97.5)	99.1 (96.6,101.5)
Body fat %	34.0 (33.3,34.7)	36.0 (34.3,37.8)	33.4 (33.0,33.8)	34.8 (33.8,35.8)	33.1 (32.8,33.5)	34.3 (33.1,35.5)
Insulin sensitivity IVGTT)((mU/L)-1 min-1) [2]	2.8 (2.4,3.1)	2.8 (2.0,3.8)	2.6 (2.4,2.8)	3.1 (2.6,3.7)	2.8 (2.6,3.1)	2.7 (2.2,3.3)
Fasting insulin (pmol/L)	96.8 (46.3,93.3)	72.9 (58.9,86.8)	77.7 (63.6,91.7)	72.1 (64.2,80.0)	61.5 (48.0,74.9)	65.9 (56.9,74.8)
Fasting glucose (mmol/L) [2]	5.6 (5.5,5.7)	5.7 (5.3,6.2)	5.6 (5.5,5.6)	5.6 (5.3,5.8)	5.5 (5.4,5.6)	5.6 (5.4,5.9)
HOMA2-IR[2]	1.2 (1.1,1.4)	1.1 (0.9,1.5)	1.3 (1.2,1.4)	1.2 (1.0,1.4)	1.2 (1.2,1.3)	1.3 (1.1,1.5)
NEFA (μmol/L)	633.6 (573.7,693.5)	649.0 (480.1,817.9)	661.4 (625.9,696.9)	668.9 (573.1,764.8)	691.9 (657.5,726.2)	650.2 (542.9,757.5)
Total cholesterol (mmol/L)	5.7 (5.5,5.8)	5.6 (5.1,6.0)	5.4 (5.3,5.5)	5.1 (4.8,5.3)	5.3 (5.2,5.4)	5.1 (4.8,5.4)
TAG (mmol/L) [2]	1.4 (1.3,1.5)	1.5 (1.1,1.9)	1.4 (1.3,1.4)	1.3 (1.1,1.6)	1.4 (1.3,1.4)	1.3 (1.1,1.6)
HDL Cholesterol (mmol/L) [2]	1.4 (1.3,1.4)	1.3 (1.2,1.5)	1.4 (1.3,1.4)	1.3 (1.2,1.4)	1.3 (1.3,1.3)	1.3 (1.2,1.4)
Apolipoprotein A1 (mg/dL)	1.2 (1.2,1.3)	1.3 (1.1,1.4)	1.2 (1.2,1.2)	1.2 (1.1,1.3)	1.2 (1.1,1.2)	1.2 (1.1,1.3)
LDL Cholesterol (mmol/L)	3.6 (3.5,3.7)	3.4 (3.1,3.8)	3.3 (3.3,3.4)	3.1 (2.9,3.3)	3.3 (3.3,3.4)	3.2 (3.0,3.5)
Apolipoprotein B (mg/dL)	1.03 (0.97,1.08)	0.98 (0.84,1.12)	0.94 (0.90,0.97)	0.85 (0.77,0.93)	0.91 (0.88,0.94)	0.89 (0.80,0.98)
Proportion of sdLDL (%)	26.1 (22.5,29.7)	31.7 (21.4,42.0)	26.2 (24.1,28.3)	20.3 (14.5,26.0)	24.4 (22.4,26.5)	26.1 (19.6,32.7)
Adiponectin (μg/mL)	10.5 (10.1,11.0)	9.8 (8.6,11.2)	10.0 (9.7,10.3)	11.0 (10.2,11.8)	10.1 (9.8,10.4)	10.0 (9.1,10.9)

Values are follow-up values adjusted for baseline values (95% CI) or [2]GM (95% CI.). LL represents subjects homozygous for the *PPARA* Leu162 allele and LV carriers of the Val162 allele.

Appendix 5.4: Table 5.4: Effect of Interaction between fat intake *PPARG* Pro12Ala genotypes and obesity measures, insulin sensitivity measures and plasma lipids and adiponectin concentrations in White subjects

	HS		HM		LF	
Phenotype	PP (*n* = 47)	PA+AA (n = 7)	PP (*n* = 109)	PA + AA (*n* = 20)	PP (*n* = 109)	PA + AA (*n* = 37)
	Follow-up (95%CI)	Follow-up (95%CI)	Follow-up (95%CI)	Follow-up (95%CI)	Follow-up (95%CI)	Follow-up (95%CI)
BMI (kg/m2)	29.0 (28.7,29.2)	28.2 (27.4,28.9)	28.8 (28.6,29.0)	27.7 (27.3,28.2)	28.4 (28.2,28.5)	27.7 (27.3,28.0)
Waist circumference (cm)	97.9 (96.7,99.2)	96.0 (92.8,99.2)	98.2 (97.3,99.0)	97.4 (95.5,99.3)	97.0 (96.1,97.8)	96.4 (95.0,97.8)
Body fat %[1]	34.7 (34.1,35.3)	32.1 (30.4,33.8)	34.0 (33.6,34.4)	31.3 (30.2,32.4)	33.8 (33.4,34.2)	30.9 (30.2,31.7)
Insulin sensitivity IVGTT)((mU/L)-1 min-1) [2]	2.6 (2.3,3.0)	2.9 (2.2,3.9)	2.6 (2.3,2.8)	2.5 (2.1,3.0)	2.8 (2.6,3.1)	3.0 (2.6,3.4)
Fasting insulin (pmol/L)[2]	68.8 (44.1,93.5)	68.3(50.4,86.1)	82.8 (66.5,99.1)	62.1 (51.5,72.7)	59.0 (42.2,74.8)	64.0 (56.3,71.8)
Fasting glucose (mmol/L)	5.6 (5.5,5.7)	5.7 (5.3,6.0)	5.6 (5.5,5.6)	5.7 (5.5,5.9)	5.5 (5.4,5.6)	5.5 (5.4,5.7)
HOMA2-IR[2]	1.3 (1.1,1.4)	1.2 (0.9,1.7)	1.3 (1.2,1.4)	1.3 (1.1,1.6)	1.3 (1.2,1.4)	1.1 (1.0,1.3)
NEFA (μmol/L)	652.5 (595.6,709.5)	584.6 (416.2,752.9)	683.0 (645.5,720.6)	686.3 (585.5,787.2)	678.1 (639.6,716.7)	717.2 (643.2,791.2)
Total cholesterol (mmol/L)	5.5 (5.4,5.7)	5.8 (5.4,6.2)	5.3 (5.2,5.4)	5.4 (5.2,5.7)	5.2 (5.1,5.3)	5.3 (5.2,5.5)
Triglycerides (mmol/L) [2]	1.4 (1.3,1.5)	1.3 (1.0,1.6)	1.3 (1.3,1.4)	1.4 (1.2,1.6)	1.3 (1.3,1.4)	1.3 (1.2,1.5)
HDL Cholesterol (mmol/L) [2]	1.4 (1.3,1.4)	1.3 (1.2,1.4)	1.3 (1.3,1.4)	1.3 (1.3,1.4)	1.3 (1.3,1.3)	1.2 (1.2,1.3)
Apolipoprotein A1 (g/L)	1.2 (1.2,1.3)	1.2 (1.0,1.3)	1.2 (1.2,1.2)	1.2 (1.1,1.3)	1.2 (1.1,1.2)	1.1 (1.1,1.2)
LDL Cholesterol (mmol/L)	3.5 (3.4,3.6)	3.9 (3.6,4.2)	3.2 (3.2,3.3)	3.4 (3.2,3.7)	3.2 (3.2,3.3)	3.5 (3.3,3.6)
Proportion of sdLDL (%)	0.99 (0.94,1.04)	1.04 (0.88,1.19)	0.90 (0.86,0.93)	0.96 (0.87,1.05)	0.89 (0.85,0.92)	0.94 (0.88,1.01)
Apolipoprotein B (g/L)	26.3 (23.2,29.5)	26.1 (18.1,34.0)	24.9 (22.8,27.0)	23.1 (18.4,27.8)	23.8 (21.7,26.0)	25.3 (21.9,28.8)
Adiponectin (μg/mL)	10.6 (10.2,11.1)	9.1 (8.0,10.2)	10.3 (10.0,10.6)	10.1 (9.4,10.9)	10.4 (10.1,10.7)	9.5 (9.0,10.0)

Values are follow-up values adjusted for baseline values (95% CI) or [2]GM (95% CI.). PP represents subjects homozygous for the *PPARG* Pro12 allele and PA + AA carriers of the Ala12 allele.

Appendix 6.1: Characteristics of subjects at baseline by randomized treatment

Table 6.1 Characteristics of subjects at baseline by randomized treatment

	Placebo n=88	0.45 g/day n=94	0.9 g/day n=93	1.8 g/day n=92
Male	34 (38.6%)	36 (38.3%)	36 (38.7%)	36 (39.1%)
Female	54 (61.4%)	58 (61.7%)	57 (61.3%)	56 (60.9%)
Age (y)	55 (54 to 57)	55 (53 to 56)	55 (54 to 56)	55 (54 to 57)
Ethnicity[1]				
White	68 (77.3%)	76 (80.9%)	73 (78.5%)	79 (85.9%)
Black	9 (10.2%)	4 (4.3%)	6 (6.5%)	1 (1.1%)
Asian[2]	6 (6.8%)	6 (6.4%)	10 (10.8%)	2 (2.2%)
Far Eastern	2 (2.3%)	4 (4.3%)	0 (0.0%)	4 (4.3%)
Other	3 (3.4%)	4 (4.3%)	4 (4.3%)	6 (6.5%)
BMI (kg/m^2)	26.2 (25.3,27.1)	25.1 (24.3,25.9)	26.2 (25.3,27.0)	25.1 (24.3,26.0)
Total-C:HDL-C[2]	3.59 (3.40, 3.79)	3.53 (3.35 , 3.73)	3.43 (3.26 , 3.62)	3.47 (3.28 , 3.68)
TC (mmol/L)	5.6 (5.4 , 5.7)	5.5 (5.3 , 5.7)	5.4 (5.2 , 5.6)	5.5 (5.4 , 5.7)
TAG(mmol/L)[3]	1.2 (1.0,1.3)	1.1 (1.0,1.3)	1.1 (1.0,1.2)	1.1 (1.0,1.2)
HDL-C(mmol/L)	1.53 (1.44 , 1.61)	1.54 (1.47 , 1.61)	1.56 (1.48 , 1.64)	1.58 (1.48 , 1.68)
LDL-C(mmol/L)	3.5 (3.3 , 3.6)	3.4 (3.2 , 3.6)	3.3 (3.2 , 3.5)	3.4 (3.2 , 3.5)
Systolic BP (mmHg)	126 (123,129)	122 (119,125)	126 (123,129)	121 (119,124)
Diastolic BP (mmHg)	76 (75,78)	73 (71,74)	76 (74,77)	73 (72,75)

Measurements made at baseline after a 4-week run-in on normal diet with placebo supplement and after 6 and 12 months during which normal diet was supplemented with (n-3) LCP at the daily doses shown. Values are n (%) or mean (95% CI) and do not differ by treatment allocation.[1]Self-reported ethnicity. [2]Asian: South Asian, SE Asian & Middle Eastern. [2]TC:HDL-C molar ratio.[3]Geometric mean of TAG.

Appendix 6.2 Plasma LDL concentrations stratified by *PPARG* Pro12Ala after n-3 LCP treatment

Table 6.2 Plasma LDL-C concentrations stratified by *PPARG* Pro12Ala after n-3 LCP treatment in all subjects

PPARG Pro12Ala	LDL-C(mmol/L)	Placebo	0.45g/day	0.9g/day	1.8g/day
Pro12 homozygotes		*n* = 46	*n* = 49	*n* = 54	*n* = 57
	Baseline	3.3 (3.0,3.5)	3.2 (3.0,3.4)	3.2 (3.0,3.4)	3.1 (2.9,3.3)
	6 months	3.5 (3.2,3.7)	3.5 (3.2,3.8)	3.4 (3.2,3.6)	3.3 (3.1,3.5)
	12 months	3.4 (3.1,3.6)	3.4 (3.2,3.7)	3.4 (3.2,3.6)	3.3 (3.1,3.6)
	Average	3.4 (3.2,3.6)	3.5 (3.2,3.7)	3.4 (3.2,3.6)	3.3 (3.1,3.5)
	Change	0.17 (0.03,0.32)	0.28 (0.09,0.47)	0.17 (0.03,0.31)	0.19 (0.06,0.32)
	Treatment effect	0.000 [Reference]	0.093 (-0.105,0.292)	0.108 (-0.081,0.296)	-0.014 (-0.207,0.178)
Ala12 carriers		*n* =13	*n* = 19	*n* = 9	*n* =16
	Baseline	3.3 (2.7,3.9)	3.6 (3.3,4.0)	3.2 (2.6,3.9)	3.3 (3.0,3.7)
	6 months	3.6 (3.2,4.0)	3.8 (3.4,4.1)	3.4 (2.4,4.3)	3.5 (3.2,3.9)
	12 months	3.5 (3.1,4.0)	3.7 (3.3,4.1)	3.2 (2.5,3.8)	3.8 (3.4,4.2)
	Average	3.6 (3.2,4.0)	3.7 (3.4,4.1)	3.2 (2.5,3.9)	3.7 (3.3,4.0)
	Change	0.31 (-0.14,0.76)	0.08 (-0.18,0.35)	0.01 (-0.34,0.35)	0.35 (-0.07,0.77)
	Treatment effect	0.000 [Reference]	-0.071 (-0.493,0.350)	-0.139 (-0.534,0.257)	0.068 (-0.363,0.498)

Table 6.3 Plasma LDL-C concentrations stratified by *PPARG* Pro12Ala after n-3 LCP treatment in White subjects

PPARG Pro12Ala	LDL-C (mmol/L)	Placebo	0.45g/day	0.9g/day	1.8g/day
Pro12 homozygotes		*n* = 38	*n* = 39	*n* = 43	*n* = 47
	Baseline	3.3 (3.1,3.6)	3.3 (3.1,3.6)	3.2 (2.9,3.4)	3.1 (2.9,3.3)
	6 months	3.4 (3.2,3.7)	3.7 (3.4,4.1)	3.4 (3.1,3.6)	3.3 (3.1,3.5)
	12 months	3.3 (3.1,3.6)	3.6 (3.3,3.9)	3.4 (3.1,3.6)	3.3 (3.1,3.6)
	Average	3.4 (3.2,3.6)	3.7 (3.4,4.0)	3.4 (3.1,3.6)	3.3 (3.1,3.5)
	Change	0.07 (-0.07,0.21)	0.35 (0.13,0.57)	0.19 (0.03,0.35)	0.23 (0.10,0.37)
	Treatment effect	0.000 [Reference]	0.279 (0.061,0.497)	0.178 (-0.031,0.388)	0.101 (-0.110,0.311)
Ala12 carriers		*n* =10	*n* = 14	*n* = 7	*n* =15
	Baseline	3.1 (2.4,3.8)	3.6 (3.1,4.0)	3.4 (2.5,4.2)	3.4 (3.0,3.7)
	6 months	3.5 (3.0,4.0)	3.6 (3.2,4.0)	3.6 (2.3,4.9)	3.6 (3.2,4.0)
	12 months	3.6 (3.0,4.2)	3.6 (3.3,4.0)	3.4 (2.6,4.2)	3.8 (3.4,4.3)
	Average	3.6 (3.0,4.1)	3.6 (3.3,4.0)	3.4 (2.5,4.3)	3.7 (3.3,4.1)
	Change	0.45 (-0.11,1.01)	0.07 (-0.20,0.34)	0.05 (-0.42,0.52)	0.33 (-0.11,0.78)
	Treatment effect	0.000 [Reference]	-0.184 (-0.682,0.313)	-0.188 (-0.627,0.251)	0.003 (-0.481,0.488)

For tables 6.2 and 6.3 plasma LDL-C concentrations (95% CI) (mmol/L) are shown. Data is presented for *PPARG* Pro12 homozygous and *PPARG* Ala12-allele carriers for all and White subjects, for whom DNA samples and plasma LDL-C measurements were available. All variables were measured at baseline after a 4-week run-in on normal diet with a placebo supplement and after 6 and 12 months, during which normal diet was supplemented with n-3 LCP at the daily doses shown. The average of the measurements at 6 and 12 months was subtracted from those made at baseline to determine the average change. Treatment effects were determined by univariate ANCOVA. *P*-values are adjusted for BMI, age, ethnicity and gender for all subjects and *P*-values are adjusted for BMI, age and gender for Whites (nominally significant at $P < 0.05$) (nominally significant at $P < 0.05$. Treatment x *PPARG* Pro12Ala $P=0.29$ for all subjects and $P=0.1$for White subjects.

Appendix 6.3 Plasma HDL concentrations stratified by *PPARG* Pro12Ala after n-3 LCP treatment

Table 6.4 Plasma HDL-C concentrations stratified by *PPARG* Pro12Ala after n-3 LCP treatment in all subjects

PPARG Pro12Ala	HDL-C(mmol/L)	Placebo	0.45g/day	0.9g/day	1.8g/day
Pro12 homozygotes		*n* = 46	*n* = 49	*n* = 54	*n* = 57
	Baseline	1.5 (1.4,1.7)	1.6 (1.4,1.7)	1.7 (1.5,1.8)	1.7 (1.6,1.9)
	6 months	1.5 (1.4,1.6)	1.6 (1.5,1.7)	1.6 (1.5,1.8)	1.7 (1.5,1.8)
	12 months	1.5 (1.4,1.6)	1.6 (1.5,1.7)	1.7 (1.5,1.8)	1.7 (1.6,1.9)
	Average	1.5 (1.4,1.6)	1.6 (1.5,1.7)	1.7 (1.5,1.8)	1.7 (1.6,1.8)
	Change	-0.03 (-0.12,0.05)	0.02 (-0.05,0.09)	-0.03 (-0.10,0.05)	0.01 (-0.06,0.08)
	Treatment effect	0.000 [Reference]	0.064 (-0.033,0.160)	-0.029 (-0.122,0.063)	0.093 (-0.001,0.187)
Ala12 carriers		*n* =13	*n* = 19	*n* = 9	*n* =16
	Baseline	1.6 (1.4,1.8)	1.6 (1.4,1.8)	1.7 (1.3,2.2)	1.5 (1.3,1.8)
	6 months	1.5 (1.4,1.7)	1.6 (1.4,1.7)	1.6 (1.2,1.9)	1.6 (1.3,1.8)
	12 months	1.6 (1.4,1.9)	1.5 (1.4,1.7)	1.6 (1.3,1.9)	1.5 (1.3,1.7)
	Average	1.6 (1.4,1.8)	1.5 (1.4,1.7)	1.6 (1.3,1.9)	1.5 (1.3,1.7)
	Change	-0.01 (-0.09,0.07)	-0.04 (-0.15,0.07)	-0.16 (-0.50,0.17)	-0.01 (-0.13,0.12)
	Treatment effect	0.000 [Reference]	-0.031 (-0.188,0.127)	-0.018 (-0.167,0.131)	-0.013 (-0.177,0.151)

Table 6.5 Plasma HDL concentrations stratified by *PPARG* Pro12Ala after n-3 LCP treatment in White subjects

PPARG Pro12Ala	HDL-C (mmol/L)	Placebo	0.45g/day	0.9g/day	1.8g/day
Pro12 homozygotes		*n* = 38	*n* = 39	*n* = 43	*n* = 47
	Baseline	1.5 (1.4,1.7)	1.6 (1.5,1.8)	1.7 (1.6,1.9)	1.7 (1.5,1.9)
	6 months	1.5 (1.3,1.6)	1.6 (1.5,1.8)	1.7 (1.6,1.8)	1.7 (1.5,1.8)
	12 months	1.5 (1.4,1.6)	1.6 (1.5,1.8)	1.7 (1.6,1.8)	1.8 (1.6,1.9)
	Average	1.5 (1.4,1.6)	1.6 (1.5,1.8)	1.7 (1.6,1.8)	1.7 (1.6,1.9)
	Change	-0.02 (-0.12,0.08)	0.01 (-0.07,0.09)	-0.03 (-0.11,0.05)	0.02 (-0.06,0.10)
	Treatment effect	0.000 [Reference]	0.062 (-0.046,0.171)	-0.030 (-0.133,0.074)	0.092 (-0.013,0.197)
Ala12 carriers		*n* =10	*n* = 14	*n* = 7	*n* =15
	Baseline	1.6 (1.3,1.8)	1.5 (1.3,1.7)	1.7 (1.2,2.2)	1.5 (1.3,1.8)
	6 months	1.5 (1.3,1.8)	1.5 (1.4,1.7)	1.5 (1.2,1.8)	1.5 (1.3,1.7)
	12 months	1.7 (1.4,1.9)	1.5 (1.3,1.6)	1.6 (1.2,2.0)	1.5 (1.3,1.7)
	Average	1.6 (1.3,1.8)	1.5 (1.3,1.6)	1.5 (1.3,1.8)	1.5 (1.3,1.7)
	Change	0.01 (-0.08,0.10)	-0.03 (-0.16,0.10)	-0.18 (-0.63,0.27)	-0.03 (-0.16,0.10)
	Treatment effect	0.000 [Reference]	-0.067 (-0.242,0.108)	-0.012 (-0.169,0.145)	-0.055 (-0.227,0.118)

For tables 6.4 and 6.5 plasma HDL-C concentrations (95% CI) (mmol/L) are shown. Data is presented for *PPARG* Pro12 homozygous and *PPARG* Ala12-allele carriers for all and White subjects, for whom DNA samples and plasma HDL-C measurements were available. All variables were measured at baseline after a 4-week run-in on normal diet with a placebo supplement and after 6 and 12 months, during which normal diet was supplemented with n-3 LCP at the daily doses shown. The average of the measurements at 6 and 12 months was subtracted from those made at baseline to determine the average change. Treatment effects were determined by univariate ANCOVA. *P*-values are adjusted for BMI, age, ethnicity and gender for all subjects and *P*-values are adjusted for BMI, age and gender for Whites (nominally significant at $P < 0.05$) (nominally significant at $P < 0.05$. Treatment x *PPARG* Pro12Ala $P=0.61$ for all subjects and $P=0.44$ for White subjects.

Appendix 6.4 Plasma HDL-C concentrations stratified by *PPARA* Leu162Val after n-3 LCP

Table 6.6 Plasma HDL-C concentrations stratified by *PPARA* Leu162Val after n-3 LCP treatment in all subjects

PPARA Leu162Val	HDL-C(mmol/L)	Placebo	0.45g/day	0.9g/day	1.8g/day
Leu162 homozygotes		*n* = 61	*n* = 73	*n* = 66	*n* = 69
	Baseline	1.6 (1.5,1.7)	1.6 (1.5,1.7)	1.7 (1.6,1.8)	1.6 (1.5,1.8)
	6 months	1.5 (1.4,1.6)	1.6 (1.5,1.7)	1.6 (1.5,1.8)	1.6 (1.5,1.7)
	12 months	1.5 (1.4,1.6)	1.6 (1.5,1.7)	1.6 (1.5,1.7)	1.7 (1.6,1.8)
	Average	1.5 (1.4,1.6)	1.6 (1.5,1.7)	1.6 (1.5,1.7)	1.7 (1.5,1.8)
	Change	-0.04 (-0.11,0.03)	0.02 (-0.05,0.08)	-0.04 (-0.12,0.04)	0.02 (-0.04,0.08)
	Treatment effect	0.000 [Reference]	0.051 (-0.032,0.133)	-0.022 (-0.102,0.059)	0.072 (-0.012,0.156)
Val162 carriers		*n* =6	*n* = 4	*n* = 7	*n* =6
	Baseline	1.7 (1.4,2.1)	1.6 (1.0,2.1)	1.6 (1.2,1.9)	1.8 (1.2,2.4)
	6 months	1.6 (1.2,1.9)	1.6 (1.1,2.1)	1.5 (1.1,1.9)	1.9 (1.7,2.2)
	12 months	1.6 (1.4,1.9)	1.6 (0.9,2.3)	1.5 (1.3,1.7)	2.0 (1.6,2.3)
	Average	1.6 (1.4,1.8)	1.6 (1.0,2.2)	1.5 (1.2,1.8)	2.0 (1.7,2.2)
	Change	-0.13 (-0.35,0.10)	0.03 (-0.32,0.37)	-0.05 (-0.20,0.10)	0.15 (-0.25,0.55)
	Treatment effect	0.000 [Reference]	0.078 (-0.151,0.306)	-0.228 (-0.458,0.002)	0.306 (0.103,0.508)

Table 6.7 Plasma HDL-C concentrations stratified by *PPARA* Leu162Val after n-3 LCP treatment in White subjects

PPARA Leu161Val	HDL-C (mmol/L)	Placebo	0.45g/day	0.9g/day	1.8g/day
Leu162 homozygotes		*n* = 49	*n* = 57	*n* = 52	*n* = 59
	Baseline	1.6 (1.5,1.7)	1.6 (1.5,1.7)	1.7 (1.6,1.8)	1.6 (1.5,1.8)
	6 months	1.6 (1.4,1.7)	1.6 (1.5,1.7)	1.7 (1.5,1.8)	1.6 (1.5,1.7)
	12 months	1.6 (1.5,1.7)	1.6 (1.5,1.7)	1.7 (1.5,1.8)	1.7 (1.5,1.8)
	Average	1.6 (1.5,1.7)	1.6 (1.5,1.7)	1.7 (1.5,1.8)	1.6 (1.5,1.8)
	Change	-0.02 (-0.11,0.06)	0.02 (-0.06,0.09)	-0.05 (-0.13,0.04)	0.01 (-0.05,0.08)
	Treatment effect	0.000 [Reference]	0.044 (-0.051,0.139)	-0.008 (-0.099,0.083)	0.052 (-0.043,0.147)
Val162 carriers		*n* =6	*n* = 4	*n* = 7	*n* =6
	Baseline	1.7 (1.4,2.1)	1.6 (1.0,2.1)	1.6 (1.2,1.9)	1.8 (1.2,2.4)
	6 months	1.6 (1.2,1.9)	1.6 (1.1,2.1)	1.5 (1.1,1.9)	1.9 (1.7,2.2)
	12 months	1.6 (1.4,1.9)	1.6 (0.9,2.3)	1.5 (1.3,1.7)	2.0 (1.6,2.3)
	Average	1.6 (1.4,1.8)	1.6 (1.0,2.2)	1.5 (1.2,1.8)	2.0 (1.7,2.2)
	Change	-0.13 (-0.35,0.10)	0.03 (-0.32,0.37)	-0.05 (-0.20,0.10)	0.15 (-0.25,0.55)
	Treatment effect	0.000 [Reference]	0.078 (-0.151,0.306)	-0.228 (-0.458,0.002)	0.306 (0.103,0.508)

For tables 6.6 and 6.7 plasma HDL-C concentrations (95% CI) (mmol/L) are shown. Data is presented for *PPARA* Leu162 homozygotes homozygous and *PPARA* Val162 carriers for all and White subjects, for whom DNA samples and plasma HDL-C measurements were available. All variables were measured at baseline after a 4-week run-in on normal diet with a placebo supplement and after 6 and 12 months, during which normal diet was supplemented with n-3 LCP at the daily doses shown. The average of the measurements at 6 and 12 months was subtracted from those made at baseline to determine the average change. Treatment effects were determined by univariate ANCOVA. *P*-values are adjusted for BMI, age, ethnicity and gender for all subjects and *P*-values are adjusted for BMI, age and gender for Whites (nominally significant at $P < 0.05$),nominally significant at $P < 0.05$. Treatment x *PPARA* Leu162Val $P=0.37$ for all subjects and $P=0.35$ for White subjects

Appendix 6.5 Plasma LDL-C concentrations stratified by *PPARA* Leu162Val after n-3 LCP

Table 6.8 Plasma LDL-C concentrations stratified by *PPARA* Leu162Val after n-3 LCP treatment in all subjects

PPARA Leu162Val	LDL-C(mmol/L)	Placebo	0.45g/day	0.9g/day	1.8g/day
Leu162 homozygotes		*n* = 61	*n* = 73	*n* = 66	*n* = 69
	Baseline	3.2 (3.0,3.5)	3.3 (3.1,3.5)	3.2 (3.0,3.4)	3.1 (3.0,3.3)
	6 months	3.5 (3.3,3.7)	3.5 (3.3,3.7)	3.4 (3.2,3.6)	3.3 (3.2,3.5)
	12 months	3.4 (3.2,3.6)	3.5 (3.3,3.7)	3.3 (3.1,3.5)	3.5 (3.3,3.7)
	Average	3.4 (3.3,3.6)	3.5 (3.3,3.7)	3.3 (3.1,3.5)	3.4 (3.2,3.6)
	Change	0.19 (0.05,0.33)	0.23 (0.10,0.37)	0.12 (0.00,0.24)	0.24 (0.11,0.38)
	Treatment effect	0.000 [Reference]	0.050 (-0.119,0.219)	0.025 (-0.140,0.189)	0.026 (-0.146,0.198)
Val162 carriers		*n* =6	*n* = 4	*n* = 7	*n* =6
	Baseline	3.9 (3.1,4.7)	2.9 (1.7,4.1)	3.3 (2.6,4.0)	3.0 (1.8,4.1)
	6 months	3.3 (2.8,3.8)	3.2 (1.7,4.6)	3.4 (2.9,3.9)	3.7 (2.8,4.5)
	12 months	4.0 (3.3,4.6)	3.2 (2.0,4.3)	3.5 (3.0,4.0)	3.4 (2.4,4.5)
	Average	3.8 (3.2,4.3)	3.2 (2.0,4.3)	3.4 (3.0,3.9)	3.6 (2.7,4.4)
	Change	-0.13 (-0.60,0.33)	0.25 (-0.68,1.18)	0.14 (-0.30,0.58)	0.60 (0.03,1.17)
	Treatment effect	0.000 [Reference]	0.027 (-0.571,0.625)	-0.359 (-0.913,0.195)	0.386 (-0.156,0.928)

Table 6.9 Plasma LDL-C concentrations stratified by *PPARA* Leu162Val after n-3 LCP treatment in White subjects

PPARA Leu161Val	LDL-C (mmol/L)	Placebo	0.45g/day	0.9g/day	1.8g/day
Leu162 homozygotes		*n* = 49	*n* = 57	*n* = 52	*n* = 59
	Baseline	3.3 (3.0,3.5)	3.3 (3.1,3.5)	3.2 (2.9,3.4)	3.1 (3.0,3.3)
	6 months	3.5 (3.2,3.7)	3.6 (3.4,3.9)	3.4 (3.1,3.6)	3.3 (3.2,3.5)
	12 months	3.4 (3.1,3.6)	3.6 (3.4,3.8)	3.2 (3.0,3.5)	3.5 (3.3,3.7)
	Average	3.4 (3.2,3.6)	3.6 (3.4,3.8)	3.3 (3.1,3.5)	3.4 (3.2,3.6)
	Change	0.14 (-0.01,0.29)	0.28 (0.13,0.43)	0.13 (0.00,0.26)	0.27 (0.12,0.41)
	Treatment effect	0.000 [Reference]	0.158 (-0.029,0.345)	0.066 (-0.114,0.246)	0.092 (-0.095,0.278)
Val162 carriers		*n* =6	*n* = 4	*n* = 7	*n* =6
	Baseline	3.9 (3.1,4.7)	2.9 (1.7,4.1)	3.3 (2.6,4.0)	3.0 (1.8,4.1)
	6 months	3.3 (2.8,3.8)	3.2 (1.7,4.6)	3.4 (2.9,3.9)	3.7 (2.8,4.5)
	12 months	4.0 (3.3,4.6)	3.2 (2.0,4.3)	3.5 (3.0,4.0)	3.4 (2.4,4.5)
	Average	3.8 (3.2,4.3)	3.2 (2.0,4.3)	3.4 (3.0,3.9)	3.6 (2.7,4.4)
	Change	-0.13 (-0.60,0.33)	0.25 (-0.68,1.18)	0.14 (-0.30,0.58)	0.60 (0.03,1.17)
	Treatment effect	0.000 [Reference]	0.027 (-0.571,0.625)	-0.359 (-0.913,0.195)	0.386 (-0.156,0.928)

For tables 6.8 and 6.9 plasma LDL-C concentrations (95% CI) (mmol/L) are shown. Data is presented for *PPARA* Leu162 homozygotes homozygous and *PPARA* Val162 carriers for all and White subjects, for whom DNA samples and plasma LDL-C measurements were available. All variables were measured at baseline after a 4-week run-in on normal diet with a placebo supplement and after 6 and 12 months, during which normal diet was supplemented with n-3 LCP at the daily doses shown. The average of the measurements at 6 and 12 months was subtracted from those made at baseline to determine the average change. Treatment effects were determined by univariate ANCOVA. *P*-values are adjusted for BMI, age, ethnicity and gender for all subjects and *P*-values are adjusted for BMI, age and gender for Whites (nominally significant at $P < 0.05$) (nominally significant at $P < 0.05$. Treatment x *PPARA* Leu162Val $P=0.46$ for all subjects and $P=0.43$ for White subjects

Appendix 7.1 EDT circular email

Circular e-mail for use for recruitment of volunteers for study ref. 08/H0805/2, approved by Bromley Research Ethics Committee. This project contributes to the College's role in conducting research, and teaching research methods. You are under no obligation to reply to this email, however if you choose to, participation in this research is voluntary and you may withdraw at any time

INVESTIGATION INTO INCORPORATION OF (N−3) POLYUNSATURATED FATTY ACIDS, EICOSAPENTAENOIC ACID AND DOCOSAHEXAENOIC ACID, INTO ERYTHROCYTE MEMBRANES AND EFFECTS ON NOVEL MARKERS OF CARDIOVASCULAR RISK

Short title: The EPA and DHA Trial

We need you to help us investigate how two different types of omega-3 fatty acids, present in fish oils (eicosapentaenoic acid, abbreviated to EPA, and docosahexaenoic acid, abbreviated to DHA) affect the health of your blood vessels.

If you are a healthy male, non-smoker and aged between 18 and 45 years, not allergic to fish and not receiving any medication for blood pressure or blood cholesterol then you may be able to help us. By taking part in this research you will get information about your cardiovascular health.

If you decide to take part you need to attend 3 visits as follows:

- SCREENING VISIT (approximately 45 min). You will be asked to attend the Nutritional Sciences Division (King's College London) for a screening visit. You will be provided with an information sheet and you will be given the opportunity to ask any questions you may have before proceeding. We will then ask you questions about your medical history and dietary habits, and will measure your height, weight, body fat, waist/hip circumference and blood pressure. We will take a small blood sample (16.5 ml / ~3 teaspoons) to assess your suitability for the study. You will be shown how to fit the 24 h blood pressure monitor which you will use to record your blood pressure for 24 h prior to each of the study visits. Breakfast will be provided afterwards.

- STUDY VISITS (approximately 2-3 h). Should you be suitable to take part, you would be asked to take 5 g of oil per day in the form of capsules which either contain olive oil or fish oil for the whole duration of the study (8 weeks). You will be asked to attend the Nutritional Sciences Division (King's College London) for a total of 2 occasions: after 2 weeks of taking the capsules (study visit 1) and at the end of the 8 weeks (study visit 2). 2-3 days before each visit we shall ask you to collect a urine sample for 24 h, and to be fitted with a blood pressure monitor which measures your blood pressure for 24 h. On the day before each of these visits, we shall ask you to avoid any strenuous exercise, to avoid foods high in fat and to fast overnight. On the day of your study visits 1 and 2, we shall weigh you and estimate your body fat content. After resting for 15 minutes, we will measure your pulse with external probes on your wrist and finger, and measure the small blood vessels in your finger using a special microscope camera. Following this we shall collect a small blood sample (47 ml / ~9 teaspoons) from a vein in your arm to measure changes in blood fats and other circulating markers of inflammation and blood vessel tone.

We shall take 111 ml of blood in total over the course of the study (including the screening visit) – this is equivalent to about 22 teaspoons of blood. We shall provide you with capsules to consume for two weeks before your first study visit (after the initial screening visit), and then you will be provided with more capsules to consume every day for the next 6 weeks until your final study visit.

The risk associated with the blood collection is small, but there may be a small amount of bruising. The capsules contain pure oils (either olive oil or fish oil), which are commonly found in the diet. There is no risk associated with the consumption of these capsules. You will be compensated for your time on completion of the study. The study has been approved by the Bromley National Research Ethics Committee (Reference No. 08/H0805/2) and will take place from July 2009 to December 2009.

Thank you for your interest. For further information please contact Sarah or Aseel (contact details below). We will provide you with a detailed information sheet and answer any question you have regarding the study.

Sarah Cottin sarah.cottin@kcl.ac.uk Aseel Al Saleh aseel.a.alsaleh@kcl.ac.uk
Or call 020 7848 4594 and ask for Sarah or Aseel.

Appendix 7.2 EDT study questionnaire

The EPA and DHA Trial

Please complete this questionnaire and return by email to sarah.cottin@kcl.ac.uk or aseel.alsaleh@kcl.ac.uk,

School of Biomedical and Health Sciences, King's College London, Franklin-Wilkins Building Room 4.46A, 150 Stamford Street

London SE1 9NN

* Please delete as applicable

NAME:

ADDRESS:

AGE:

D.O.B:

GENDER:

HEIGHT:

WEIGHT:

TELEPHONE NUMBER:

Email:

GP Name and Address:

Have you recently taken part in any other trial? YES / NO*

Do you smoke? YES / NO*

Do you drink alcohol? YES / NO*

If so, how many units per /week?
1 unit = glass of wine, shot of spirits, 1/2 pint of beer ……. Units day / week*

Do you suffer from any allergies? YES / NO*

If so please give details:

Do you suffer from any medical problems? YES / NO*

If so please describe:

Are you taking any medication? YES / NO*

If so please give details:

Do you take any dietary supplements? YES / NO*

How frequently do you consume oily fish? >1 portion per wk/1 portion per wk/<1 portion per wk
*(*explain what oily fish are*)*

Do you have a history of any of the following?

High blood pressure YES / NO*

Diabetes mellitus YES / NO*

Myocardial infarction-heart attack/angina/stroke/thrombosis YES / NO*

Cancer YES / NO*

Liver or gastrointestinal disorders YES / NO*

Are you receiving medication for any of the following?

Raised blood cholesterol YES / NO*

High blood pressure YES / NO*

Immune system (anti-histamines, anti-inflammatory drugs) YES / NO*

Do you have any special dietary requirements?

Lacto-ovo vegetarian/vegan/other (specify) YES / NO*

Religious dietary requirements (specify) YES / NO*

Other YES / NO*

Do you suffer from any medical problems? YES / NO*

If so please describe:

Suitability

Suitable Yes No

If Yes, book screening visit

Date ……. Time……

ID code EDT ☐ ☐ ☐ ☐

Investigator Signature …… Date ……..

Relevant information added to database ☐

Signature …….. Date

Appendix 7.3 Information sheet for participants

REC Protocol Number 08/H0805/2

YOU WILL BE GIVEN A COPY OF THIS INFORMATION SHEET

The EPA and DHA trial

We would like to invite you to participate in this original research project. You should only participate if you want to; choosing not to take part will not disadvantage you in any way. Before you decide whether you want to take part, it is important for you to understand why the research is being done and what your participation will involve. Please take time to read the following information carefully and discuss it with others if you wish. Ask us if there is anything that is not clear or if you would like more information.

What is the purpose of this study?

The omega-3 fatty acids found in oily fish are of great interest as a higher dietary intake of these omega-3 fatty acids has been shown to decrease the risk of fatal coronary heart disease (CHD) and stroke. The mechanisms by which omega-3 fatty acids found in oily fish may offer protection are yet to be explained, and it is not known whether the different types of omega-3 fatty acids differ in their effects in the body or whether they act in the same way. The main types of omega-3 fatty acids found in oily fish are *eicosapentaenoic acid (EPA)* and *docosahexaenoic acid (DHA)*. This research project is a randomised controlled trial that compares capsule intakes of different mixtures of omega-3 fatty acids equivalent to 6 portions of oily fish each week on measurements that are related to cardiovascular disease risk. Study participants will be aged 18-45 years.

Why have I been chosen?

You have been contacted as you have expressed an interest in our research. In order to participate in this study you need to be able to say 'Yes' to the following:

- I am male
- I am aged between 18 and 45 years
- I do not smoke
- I have never had a heart attack, stroke, high blood pressure (>160/90mm Hg), liver diseases, diabetes, chronic gastrointestinal disorder or cancer
- I do not have a history of excess alcohol intake or substance abuse
- I am prepared to take 5 capsules a day for 8 weeks

What will happen to me if I take part?

If you would like to participate you would first need to complete a screening questionnaire with us over the telephone (approx. 10 mins.), after which potentially eligible volunteers will be invited to attend a clinic screening appointment (approx. 45 mins) in the Metabolic Unit on 4th Floor, Corridor B, Franklin-Wilkins Building,

Stamford Street, SE1 9NH. Volunteers will need to attend this visit after an overnight fast.

The study will be explained in detail and you will be able to ask any questions you may have to ensure you will be giving fully informed consent. Following the signing of the consent form, your height, weight, waist/hip circumference and percentage body fat measurements will be recorded, and a fasting blood sample (approx. 16.5mls/ ~3 teaspoons) will be taken to determine whether liver function, haematology, blood glucose and blood lipids are within normal ranges. Seated blood pressure will be measured using an automated sphygmomanometer (blood pressure monitor) that conforms to the recommendation of British Hypertension Society. Refreshments will be provided once all samples and measurements have been made.

Eligible subjects will be asked to complete an 8-week period taking 5g oil each day in capsules (either 10 x 500mg or 5 x 1g) whilst following dietary advice to avoid oily fish intake (e.g. salmon, herring, fresh tuna, mackerel, trout, or sardines) as well as fish oil/cod liver oil supplements. You can still eat as much white fish (e.g. cod, plaice, hake, etc.), tinned tuna and shellfish as you choose. We will give you a detailed list of types of fish to avoid. You will be randomly allocated to 1 of 3 groups: 1 group will take olive oil capsules, 1 group will take fish oil capsules which contain more EPA than DHA, and the other group will take fish oil capsules which contain more DHA than EPA. You will have an equal chance of being allocated to any one of these groups.

During the intervention, you will be asked to attend the Metabolic Unit at the Franklin-Wilkins Building twice, after 2 weeks and in the end of the intervention. The week before each visit, you will be given urine bottles and an ambulatory blood pressure monitor to use 2 or 3 days before your next study visit; and we will explain in detail how to collect your urine for 24 h and how to use the blood pressure monitor.

At the end of the 2-week period you will attend the Metabolic Unit at the Franklin-Wilkins Building for your first study visit, bringing with you the collected urine and blood pressure monitor which you will have used over 24 h. We will make measurements to assess the functioning of the large and small blood vessels in your wrist and finger and take a blood sample (approx. 47 ml/ ~9 teaspoons. This visit lasts 2 to 3 h and will be repeated at the end of the intervention period. See figure 1 for an outline of these visits. To standardise everyone before these visits you will be asked to avoid strenuous physical activity, foods high in fat, caffeine or alcohol on the day before the visit. We will give you a list of foods to avoid and a list of suggested evening meals to help you with this. Only water will be allowed for 12 h before the scheduled study visit.

The overall study is shown in a diagram below:

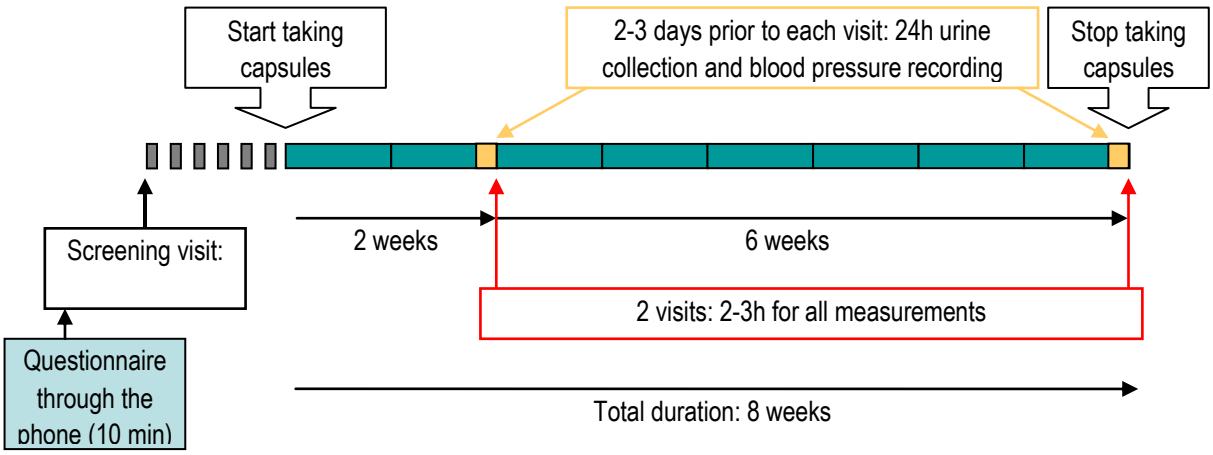


Figure 1. Study outline.

Screening visit:

1) You should avoid eating or drinking anything, except water, for 12 h before your scheduled screening visit.
2) The visit will last approximately 45 min.
3) We will give you a copy of this information sheet, explain to you all the details of the study and answer any questions you have. If you are still happy to take part in the study, you will be asked to sign a consent form.
4) We shall ask you questions about your medical history, your food habits through a Food Frequency Questionnaire and measure your weight, height, percentage body fat, blood pressure and waist and hip circumference.
5) We will need to take a small blood sample (16.5 ml /~ 3 teaspoons) to check that your blood chemistry is normal.
6) Then you will be provided with breakfast.
7) The results of the screening blood test will be given to you. If any abnormal results are found we will inform you immediately and we will provide you with a letter for your GP, which we will advise you to give to your GP.

Main study:

1) Following screening, if your results comply with the study inclusion criteria you will be invited to attend the Metabolic Unit in the Franklin-Wilkins Building on 2 further occasions; each of these visits will take approximately 2-3 h each.
2) We shall ask you to avoid eating oily fish and consuming fish oil supplements for 2 weeks prior to the start of the study and during the study. We will provide you with study capsules containing a test oil and ask you to consume 5 of these capsules per day. We will also ask you to avoid fatty foods, drinking alcohol, and any strenuous exercise the day prior to each visit to the Department of Nutrition & Dietetics.
3) We shall also ask you not to consume caffeine from midday the day before each visit and to avoid eating or drinking anything, except water, for 12 h before your scheduled study visit.

4) 2 to 3 days prior to each of the 2 remaining visits, you will be asked to collect urine samples and to record your blood pressure (using the ambulatory blood pressure monitor) for 24 h.

5) You will be asked to report to the Metabolic Unit in the Department of Nutrition & Dietetics between 08:00 h and 11:00 h, in the fasted state (i.e. without having consumed breakfast and without having consumed any food or drink for 12 h, apart from water). Make sure you drink some water on the morning of the study to avoid dehydration.

6) At each of the 2 visits (at 2 wk and 8 wk), we will then measure your blood pressure and the function of your large and small blood vessels. We shall make measurements of blood pressure using a sphygmomanometer, in which a cuff will be placed around your arm and will be inflated. The cuff causes a tingling sensation in your arm, but does not cause pain. The measurements we make to assess the function of your larger blood vessels are carried out using probes that are gently placed on your wrist and finger to determine the elasticity or tone of your arteries. The measurement of small blood vessel function involves you placing the top of your finger under a camera which can image individual capillaries (the smallest type of blood vessel) to monitor the capillary blood flow before and after inflation of a cuff placed around your forearm. All of these measurements are non-invasive and will not cause any discomfort. In the end of the visit, we will take a small sample of blood: 47 ml /~9 teaspoons.

7) Finally, following these measurements you will be offered refreshments.

After the second visit (at 2 wk), you will be provided with more oil capsules for the remainder of the study and you will continue to take 5 capsules per day for 6 weeks. You will be advised to avoid oily fish and to abstain from taking fish oil supplements or cod liver oil for the duration of the study (8 weeks in total).

Will my participation be kept confidential?

Any information collected about you during this research will be kept strictly confidential. Your GP will not be told that you are taking part in the study, unless you request us to do so. Subject confidentiality and anonymity will be observed throughout the study by use of subject codes in place of names, and the storage of subject details in a secure place. Only the investigators have access to this data. Should you wish to find out the results of this study you are welcome to contact Dr Wendy Hall (details below) for a copy of the final report once the study is finished.

What will happen to my study results?

We hope to publish the results of the whole study in a scientific journal. You will not be identified in any publication. We will be happy to discuss the overall results with you when the study is completed, and will let you know how you can get a copy of the published results if you wish.

Who is organising and funding the study?

The study is organised and funded by the Nutritional Sciences Division, Kings College London. In recognition of your time commitment, you will be paid an honorarium of £50 upon completion of the study.

Do I have to take part?

It is up to you to decide whether to take part or not. If you do decide to take part you will be given this information sheet to keep and be asked to sign a consent form. You can withdraw from the study at any time by informing one of the researchers and you are not obliged to give a reason. A decision to withdraw at any time, or a decision not to take part, will not affect the standard of care you receive.

If you decide to take part, please let us know if you have been involved in any other study in the last year.

If this study has harmed you in any way you can contact King's College London using the details below for further advice and information:

Thank you for your interest.

For further information, please contact:

Sarah Cottin, email: sarah.cottin@kcl.ac.uk

or Aseel Al Saleh, email: aseel.a.alsaleh@kcl.ac.uk

Nutritional Sciences Division, King's College London, Franklin Wilkins Building, 150

Stamford Street, London, SE1 9NH

Chief Investigator: Dr Wendy Hall Tel. 020 7848 4197 wendy.hall@kcl.ac.uk

Co-investigators: Prof Tom Sanders Tel. 020 7848 4273 tom.sanders@kcl.ac.uk; Dr Zoe Maniou Tel. 020 7848 4546 zoitsa.maniou@kcl.ac.uk

Appendix 7.4 EDT booklet

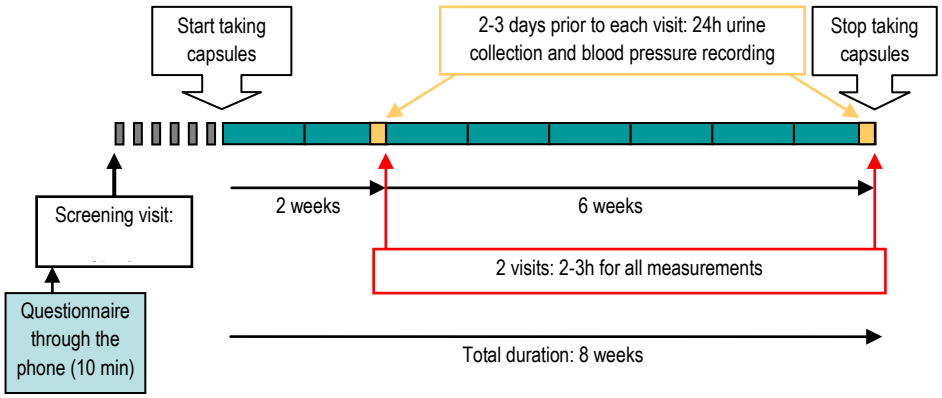
The EPA and DHA trial

A high intake of fish oils, as observed in the Inuit population, is associated to a reduced risk of heart diseases and insulin resistance. Fish oils are composed of two types of omega-3 fatty acids: eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). The beneficial effects of fish oils have been well established and omega 3 supplements have flourished on the market over the past decade. However, it remains unclear if EPA, DHA or a combination of both are required to improve insulin sensitivity and cardiovascular health.

This study aims at comparing the effect of EPA and DHA on different biological processes involved in the development of cardiovascular diseases, including inflammation, thrombosis and endothelial function.

STUDY OUTLINE

The nutritional intervention (outlined below) lasts 8 weeks during which you will be asked to take 5g of oil per day in the form of capsules containing one type of fat (either EPA, DHA or olive oil as a placebo). You will be required to attend the Metabolic Unit at the Franklin-Wilkins Building, Waterloo Campus, of King's College London on 3 different occasions: one before (screening) and 2 during the intervention.



THE DAY BEFORE THE VISITS

Prior to each of the 3 visits (screening or intervention) you should avoid having caffeine from midday, and also avoid doing exercise and drinking alcohol for 24h. You will be asked to have a low fat meal in the evening (instructions will be given) fast overnight (no food or drink, except water, from 10pm, and no breakfast on the study day). You will then attend the Metabolic Unit at a pre-arranged time between 8.30 and 10.30 am. It is important that you drink water before attending.

FIRST VISIT: SCREENING (45 min)

The purpose of this screening session is to determine whether you are suitable to participate in our study. This involves measurement of your height, weight, waist circumference, body fat and blood pressure. It also involves the collection of a blood sample to determine your blood fats and glucose levels. We will give you more information and answer any question you might have regarding the study. Breakfast will be provided In the end of the screening session.

2 VISITS DURING THE INTERVENTION (2-3H)

You will be required to attend the Metabolic Unit at the Franklin-Wilkins Building, Waterloo Campus, of King's College London after 2 weeks and in the end of the intervention (8 weeks).

2 or 3 days prior to each visit, you will be asked to collect your urine and to record your blood pressure for 24h: A cuff will be put on your left arm and inflate every half an hour during the day and every hour at night.

Each visit will consist in non-invasive vascular measurement (sensors will be placed on your finger and your wrist) and blood taking. Lunch will be provided in the end of each visit.

FISH OILS RESTRICTION

We shall ask you to avoid eating oily fish, such as salmon, trout or mackerel, and consuming fish oil supplements for the 8 week intervention period. However, you will still be allowed to eat non oily fish such as cod, haddock or sea bass. A more detailed list of fishes allowed/to avoid will be provided.

STUDY DATES

Please choose the date you will start the intervention, taking into account your availability for the 2 following visits.

	Date	Time
Day starting taking capsules (time 0)		-
2 week point		
8 week point		

WHAT YOU WILL GET BY TAKING PART

- Information on your blood fats and glucose levels
- A full biochemical screening
- Information on your body composition, physical measurements, vascular health and blood pressure
- £50 for your time

By participating in our study you will provide us with valuable information. Your commitment to our study is greatly appreciated.

Thank you

CONTACT DETAILS:

Aseel Alsaleh aseel.alsaleh@kcl.ac.uk,

Sarah Cottin sarah.cottin@kcl.ac.uk,

Appendix 7.5 List of oily fishes to avoid during the 8 weeks of intervention

Restriction in oily fish consumption

You are required to avoid any source of fish oils, including supplements and oily fishes (left of the table below) during the 8 week intervention period

Oily / fatty fish to avoid	White / non-oily fish allowed
Salmon	Cod
Trout	Haddock
Mackerel	Plaice
Herring	Coley
Sardines	Whiting
Pilchards	Lemon sole
Kipper	Skate
Eel	Halibut
Whitebait	Rock Salmon/Dogfish
Tuna (fresh only)	Ayr
Anchovies	Catfish
Swordfish	Dover sole
Bloater	Flounder
Cacha	Flying fish
Carp	Hake
Hilsa	Hoki
Jack fish	John Dory
Katla	Kalabasu
Orange roughy	Ling
Pangas	Monkfish
Sprats	Parrot fish
	Pollack
	Pomfret
	Red and grey mullet
	Red fish
	Red Snapper
	Rohu
	Sea bass
	Sea bream
	Shark
	Tilapia
	Turbot
	Tinned tuna
	Marlin

Ref: http://www.food.gov.uk/news/newsarchive/2004/jun/oilyfishdefinition

Appendix 7.6 EDT consent form

CONSENT FORM FOR PARTICIPANTS IN A NUTRITIONAL STUDY

Please complete this form after you have read the Information Sheet and you are satisfied that the research has been fully explained.

Title of Study: The EPA and DHA Trial

Research Ethics Committee Ref: 08/H0805/2

Thank you for considering taking part in this research. The person organizing the research must explain the project to you before you agree to take part.

If you have any questions arising from the Information Sheet or explanation already given to you, please ask the researcher before you decide whether to participate. You will be given a copy of this Consent Form to keep and refer to at any time.

- I confirm that I fit into the following criteria

  - I am a male aged between 18 and 45 years and do not smoke
  - I do not have a history of heart disease, stroke, high blood pressure, diabetes, thrombosis, liver disease, chronic gastrointestinal disorders or a cancer diagnosis (except basal cell carcinoma)
  - I do not have a history of excess alcohol intake or substance abuse

- I understand that if I decide at any other time during the research that I no longer wish to participate in this project, I can notify the researchers involved and be withdrawn from it immediately.
- I consent to the processing of my personal information for the purposes of this research study. I understand that such information will be treated as strictly confidential and handled in accordance with the provisions of the Data Protection Act 1998.
- I agree that the research team may use my data for future research and understand that any such use of identifiable data would be reviewed and approved by a research ethics committee. Please note that in such cases, as with this project, confidentiality and anonymity will be maintained ant it will not be possible to identify you from any publication

Participant's Statement:

I _____

agree that the research project named above has been explained to me to my satisfaction and I agree to take part in the study. I have read both the notes written above and the Information Sheet about the project, and understand what the research study involves.

Signed _____ Date _____

Investigator's Statement:

I _____

confirm that I have carefully explained the nature, demands and any foreseeable risks (where applicable) of the proposed research to the volunteer.

Signed _____ Date _____

Appendix 7.7 KCL standard venepuncture procedure

Before taking a sample, ensure the volunteer understands the procedure and is in a comfortable position. Be aware if the volunteer has ever felt faint when giving blood on previous occasions. Only appropriately trained personnel may perform venepuncture. Prior to starting the procedure, ensure that adequate hand washing takes place. Place the tourniquet around the arm, and then clean the selected site with an antiseptic swab to decontaminate the skin.

The vacutainer method (Beckton-Dickinson) is used when smaller blood volumes are required, as it reduces the risk of spillages. Screw the needle into the disposable vacutainer holder, unsheathe the needle and then insert the needle into the brachial vein with minimal pressure. Attach the vacutainer, and release the tourniquet once blood begins to flow into the vacutainer. This will facilitate a natural flow as the blood sampling progresses. Extra care should be taken when using a needle and syringe to avoid spillages when decanting into the blood tubes.

Serum samples should be collected first, followed by citrate samples (for haemostatic factors) followed by remaining samples with anticoagulants. A minimal use of the tourniquet (released during filling off the first vacutainer) is crucial to avoid platelet activation. Therefore, the last sample to be collected should be the citrate 4.5 ml, as it will be used for platelet-monocyte aggregation technique. After removing the needle, the volunteer should be asked to apply pressure to the site with a cotton wool ball, until such time that the bleeding stops. Before applying a plaster to the site, check that the volunteer is not allergic to them.

Needles must never be re-sheathed and after use they should immediately be discarded into a sharps bin.

Appendix 7.8: Blood handling protocol

Order Draw	SAMPLE	TUBES	ICE/ RT	CENTRIFUGE	SEPARATION		ANALYTES	Labels	ANALYSIS	STORAGE/ TRANSPORT
					ul / vial	vials				
4	4	4 ml fluoride oxalate (grey)	ICE	15m X 1300g @ 4OC	3	1	Glucose	Glc	KCL– ILAB	-40 o C
					3	1	Spare	Sp		-40 o C
1	8.5	8.5 ml serum	RT	15m X 1300g @ 4OC	200	1	Adiponectin	Adpn	KCH	-80 o C
					200	1	CRP	CRP	KCH	-40 o C
					200	1	Resistin	Res	KCH	-40 o C
					200	1	spare	Sp1		-40 o C
2	8.5	8.5 ml serum	RT	15m X 1300g @ 4OC	200	1	sdLDL	sdLDL		-80 o C
					200	1	TAG, NEFA, cholesterol, HDL	FLIP	KCL - ILAB	-80 o C
					200	1	MMP-9	MMP9		-80 o C
					500	1	NOx	NOx	KCL	-80 o C
					200	2	Infl markers (IL-6, IL-1...)	IF		-80 o C
					200	2	Spare	Serum Sp2,3		-80 o C
					200	1	Apolipoprotein B100	ApoB100	KCH	-80 o C
					200	1	Apolipoprotein A1	ApoA1		-80 o C
3	4	4 ml for	ICE	15m X 1300g @	300	1	Insulin	Ins	KCH	-40 o C

Order Draw	SAMPLE	TUBES insulin (LH)	ICE/ RT	CENTRIFUGE 4OC	SEPARATION		ANALYTES	Labels	ANALYSIS	STORAGE/ TRANSPORT
					300	1	Spare	Sp		-40 o C
8	4.5	4.5 ml EDTA	ICE	15m X 1300g @ 4OC	500	1	PFA	PFA	KCL - GLC	-40 o C
					600	1	prostacyclin	PGI2	KCL	-80 o C
					200	2	spare	EDTA Sp1,2		-80 o C
7	4.5	4.5 ml EDTA	ICE	Before centrifugation	~1000		EPC	EPC	KCL	Fresh WB
				15m X 1300g @ 4OC	pellet		Erythrocyte lipids + spare	Er Lip1,2		Fresh cells/ -80
					500	1	spare	EDTA Sp3		-80 o C
5,6,9	13.5	3 *4.5 ml Citrate (blue)	ICE	15m X 1300g @ 21OC	2000	1	isoprostane	IsoPs	KCL	-80 o C
					2000	1	Spare	CitSp1		-80 o C
			RT	Before centrifugation	~500		PMA	PMA	KCL	Fresh WB
				15m X 1300g @ 21OC	500	2	spare	CitSp2,3		-80 o C

Appendix 7.9 Instructions for taking a 24-hour urine collection

2 days prior to each intervention please collect all urine passed during a period of 24 hours. We will provide you with some plastic containers and plastic bags to keep the containers in. After you have made the 24-hour collection, keep it in a cool place until your appointment.

Please follow these instructions:

On the first morning when you awake, empty your bladder into the toilet. Please make a note on the collection container of the date and time you did this. After this, collect and store in the container all urine you pass during the day and the following night.

When you feel the need to have a bowel movement, first try to pass urine into the container (that is, try not to pass urine directly into the toilet with a bowel motion).

The following morning when you awake, at the same time as on the previous day (that is 24 hours after you started) complete the collection by emptying your bladder into the container. Make a note of the time you complete the collection, if this is not exactly 24 hours later don't worry.

Keep the top of the collection container closed, and keep the container in the plastic bag provided in the fridge. If you pass urine but forget to collect it, please tell us when you hand your collection in, and if possible try to estimate how much was lost. You can do this by noting how much you collect in the jug on the next occasion. Some people find it useful to pin their pants to their outer clothes with a safety pin as a reminder to make a collection each time they go to the toilet.

Please ask us if you have any queries and let us know if you have any difficulties in making the collection.

Note: Please empty your bladder in the morning before you make the first collection and please ensure you collect urine when you empty your bladder the following morning.

Contact Sarah Cottin, Email: sarah.cottin@kcl.ac.uk or Aseel Al-Saleh, Email: aseel.alsaleh@kcl.ac.uk,

Telephone: 020 7848 4594

Appendix 7.10 24 hour Blood Pressure Monitoring - Subject Information Sheet

The aim of this test is to monitor what is happening to your blood pressure over a 24 hour period. Please make your measurement 2 or 3 days before your visit to FWB. Please try to make the measurement over 25h, as the first hour when you are getting used to wearing the monitor does not always give us a representative reading. You will be asked to do your measurement at approximately the same time before each visit.

We will provide you with a diary card to fill in on the day of measurements, please fill in your activity level at the time of measurement, the time you go to bed, the time you get up and any unusual circumstances.

Procedure

Night and day; the blood pressure cuff has two settings; a day time setting and a night time setting. The monitor will automatically change to night time settings at 10pm and day time settings at 7am.Frequency of cuff inflation; During the day time the cuff will inflate every 30 minutes, during the night time the cuff will inflate every 1 hr.

During a measurement

To avoid incorrect results the arm must be kept still during measurements.

If you are standing; let your arm hang loosely, whilst keeping it still.

If you are sitting; rest your arm loosely on a table or let your arm hang loosely, whilst keeping it still.

Avoid opening and closing your hand during the measurement, and do not move your fingers.

Ensure the air tube is not kinked while the measurement is being taken.

If you are driving when a measurement starts, continue to drive normally and do not worry about keeping the arm still (please make a note on the diary card if this happens), or turn the monitor off whilst you are driving.

While you are asleep, place the recorder on its side so that the air tube will not kink.

In the event of failed measurements the monitor will repeat the measurement.

Between measurements:

Engage in normal activities.

Check that the yellow mark is still in position as the cuff may move during the day.

Using the blood pressure monitor

How to put the cuff on upper arm

Put the cuff on the upper left arm so that the position of the yellow marking of the cuff is on the artery; the opposite side to your elbow. The cuff should be wrapped on the arm tightly, but still allow a finger to slide between the cuff and the arm.

How to turn the monitor on and off

To start the 24-hr measurement press and hold the black ‘AUTO ON/OFF’ button until it bleeps and the letter ‘A’ appears on the display.

To turn the monitor off or pause it when you take the cuff off (during bathing or exercise) press and hold down the ‘AUTO ON/OFF’ button until the letter ‘A’ is no longer on the display. When you want to restart it press and hold the black ‘AUTO ON/OFF’ button until it bleeps and the letter ‘A’ appears on the display.

To deflate the cuff or terminate a recording press the red START/STOP button.

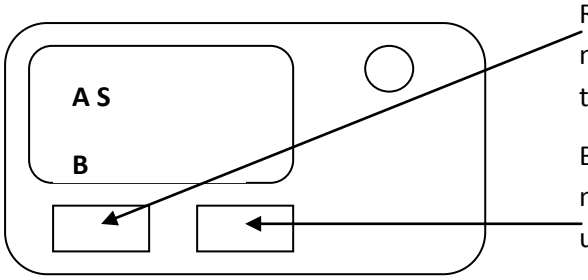
If the letter ‘E’ appears on the display at any time this means that there is an error and you will need to contact Aseel or Sarah. If the letter ‘B’ appears on the display at any time this means that the batteries need replacing.

If water gets into the unit, please remove the cuff and do not continue using the recorder. Turn off the unit and remove the batteries.

If the letter ‘E’ appears on the display at any time this means that there is an error and you will need to contact Aseel or Sarah. If the letter ‘B’ appears on the display at any time this means that the batteries need replacing.

If water gets into the unit, please remove the cuff and do not continue using the recorder. Turn off the unit and remove the batteries.

Some notes on operation:



Red START/STOP button - can be used during a measurement to terminate a measurement and deflate the cuff

Black AUTO ON/OFF button – this will switch the monitor on to automatic mode (press for 3 seconds, until the letter ‘A’ appears on the display) or to

A - This is displayed when the unit is in automatic measurement mode

B – This is displayed when battery capacity is low

S – This is displayed when the unit is in sleep interval measurement mode

IF YOU EXPERIENCE ANY PAIN OR AN EXTREMELY UNPLEASANT SENSATION DURING MEASUREMENTS PLEASE TURN OFF THE UNIT IMMEDIATELY, USING THE ON/OFF KEY AND REMOVE THE CUFF.

In case of any problems please call Aseel or Sarah on 020 7848 4594 or in

For emergency 07789002228 or 07964919960.

Diary Card

Name: Date:

Please fill in your activity level at the time of measurement, the time you go to bed, the time you get up and any unusual circumstances.

Time you go to sleep………… Time you wake up…………….

Did you take any exercise Y / N If Yes at what time…………….

Time	Activity level (please circle)			Other
	Lying down	Sitting	Walking	
	Lying down	Sitting	Walking	
	Lying down	Sitting	Walking	
	Lying down	Sitting	Walking	
	Lying down	Sitting	Walking	
	Lying down	Sitting	Walking	
	Lying down	Sitting	Walking	
	Lying down	Sitting	Walking	
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	Lying down	Sitting	Walking	
	Lying down	Sitting	Walking	

	Lying down	Sitting	Walking	
	Lying down	Sitting	Walking	
	Lying down	Sitting	Walking	
Time	Activity level			Other
	Lying down	Sitting	Walking	
	Lying down	Sitting	Walking	
	Lying down	Sitting	Walking	
	Lying down	Sitting	Walking	
	Lying down	Sitting	Walking	
	Lying down	Sitting	Walking	
	Lying down	Sitting	Walking	

Notes:

Brief Instructions: Refer to the **24 hour Blood Pressure Monitoring - Subject Information Sheet** for full instructions. During the day time (7am – 10pm), the cuff will inflate every 30 minutes and during the night time (10pm-7am) every 1 hr. Between measurements engage in normal activities, also check that the yellow mark is still in position as the cuff may move during the day. The arm must be kept still during measurements. To start the 24-hr measurement press and hold the black 'AUTO ON/OFF' button until it bleeps and the letter 'A' appears on the display. To turn the monitor off or pause it whilst bathing etc. press and hold down the 'AUTO ON/OFF' button until the letter 'A' is no longer on the display. To deflate the cuff or terminate a recording press the red START/STOP button.

In case of any problems please call Aseel or Sarah on 020 7848 4594.

IF YOU EXPERIENCE ANY PAIN OR AN EXTREMELY UNPLEASANT SENSATION DURING MEASUREMENTS PLEASE TURN OFF THE UNIT IMMEDIATELY, USING THE ON/OFF KEY AND REMOVE THE CUFF.

Appendix 7.11 Reminder e-mail

Dear …,

This is a reminder that your screening appointment for the following study is at …am tomorrow morning.

You will need to arrive at the metabolic unit on the 4th floor of Franklin-Wilkins building at the time stated above. There will be arrow signs on the 4th floor to guide you. Please use the phone at the end of corridor A to ring the unit on extension 4304 and 4612 when you arrive, and we will come and get you.

Please remember to arrive fasted, having not consumed any food or drink, except water, from 10pm tonight. We strongly advice that you drink water before you arrive to avoid dehydration.

Thank you again for participating in the study, we will look forward to seeing you tomorrow.

Sarah and Aseel

Appendix 7.12 Participant record sheet

PARTICIPANT CODE			
PARTICIPANT NAME			
D.O.B.			
DATE		TIME	
VISIT	1 / 2 /	INTERVENTION	A / B / C /
PARTICIPANT WEIGHT (Kg)			
BODY COMPOSITION TAKEN			YES / NO
CHECK	Low fat meal last night (< 10 g fat) Fasted since 10 pm last night No caffeine since midday yesterday No alcohol all day yesterday No exercise all day yesterday and today No medications taken since the beginning of the study No supplements taken since the beginning of the study Compliance to oily fish consumption restriction Compliance to capsules intake 5/d Advise to sip water		YES / NO YES / NO YES / NO YES / NO YES / NO YES / NO YES / NO YES / NO YES / NO
Blood taken 47ml	Order of draw: 1. 8.5 ml serum (Adpn...) 2. 8.5 ml serum (sdLDL, Nox, IF...) 3. 4 ml LH 4. 4 ml LX 5. 4.5 ml Citrate (IsoP) 6. 4.5 ml Citrate (IsoP) 7. 4.5 ml EDTA (EPC) – RELEASE TOURNIQUET 8. 4.5 ml EDTA (PGI2) 9. 4.5 ml Citrate (PMA)		YES / NO
Urine sample collected			YES / NO
24h Blood pressure and diary card	Collected: Data transferred: Start: … : … Sleep: … : … to … : …		YES / NO YES / NO Finish: … : …
Seated blood pressure (mmHg) To be taken at 2-5 minute intervals	Reading 1: (S) Pulse: (D) Reading 2: (S) Pulse: (D) Reading 3: (S) Pulse: (D) Average of reading 2 & 3: (S) Pulse: (D)		

Supine position:

DVP	1	2	3	Average
HR (bpm)				
SI (m/s)				
RI (%)				
PPT (ms)				

PWA	1	2	3	Average
SBP(mmHg)				
DBP(mmHg)				
MAP				
HR(bpm)				
CSp(mmHg)				
CDp(mmHg)				
Peripheral AIx %				
Central AIx %				
P1 (mmHg)				
T1 (ms)				

CAM1

ALL PICTURES AND VIDEOS HAVE TO BE RECORDED, CALCULATIONS MAY BE DONE LATER IN THE DAY

1. Pictures 1, 2, 3

Save as: yymmdd_CodeSubject_pic1 to 5 (ex: 090810_EDT01DLV1_pic1)

	Pic1	Pic2	Pic3	Pic4	Pic5	Average
CD (%)						

2. Videos 1, 2, 3 (far from the nail fold) ~10s:

Save as: yymmdd_CodeSubject_video1 to 3 (ex: 090810_EDT01DLV1_video1)

	Video 1	Video 2	Video 3	Average
FCD (%)				
FCF				

3. Doppler

Save as: yymmdd_CodeSubject_doppler1 to 6 (ex: 090810_EDT01DLV1_doppler1)

	Doppler 1	Doppler 2	Doppler 3	Average
RBCV rest – far (um/s)				
RBCV rest – close (um/s)				

4. Videos 4, 5, 6A (next to the nail fold) ~10s:

Save as: yymmdd_CodeSubject_video4, 5, and 6A (ex: 090810_EDT01DLV1_video6A)

		Video 4	Video 5	Video 6A	Average
Diameters (µm)	1. AF 1. AP 1. EF				
	2. AF 2. AP 2. EF				
	3. AF 3. AP 3. EF				
FCD (%)					
FCF (mm/s)					
1. RBCV (mm/s)					
2. RBCV (mm/s)					
3. RBCV (mm/s)					

5. Video 6B (post occlusion) ~20s

SAMEA AREA AND CAPILLARIES AS VIDEO 6A

Save as: yymmdd_CodeSubject_video6B (ex: 090810_EDT01DLV1_video6B)

	Video 6A
FCD (%)	
FCF (mm/s)	
1. RBCV (mm/s)	
2. RBCV (mm/s)	
3. RBCV (mm/s)	

* Additive to be added

Appendix 7.13 Step by step for taking a DVP and PWA measurements:

Select or enter a new patient:

For subjects on their first visits, touch the "patient" icon to enter the patient database then touch "Add" to enter the new subject's details.

For subjects on their second visit, search for this subject by adding the ID or name. Once selected, the patient's name will appear at the bottom of the screen.

Take a DVP measurement:

From the main menu, select the protocol, by touching the icon and click OK to start the test. The spot check test screen will be displayed. The signal displayed might be small at first but they will gradually increase. The unit will automatically perform three tests and display the averaged results. If the tests are outside of 20 per cent of their average, you will be asked either to repeat a particular test, or to repeat the protocol if the all the tests were very variable. Select "Accept" or "Reject" and save the data.

Step by step for taking a PWA measurement:

1-Select or enter a new patient:

For subjects on their first visits, open the subject screen by clicking on the "patient" button. This screen will allow you to create a new subject entry by clicking the "Create New" button and enter the subject's details.

For subjects on their second visit, select an existing subject from the database by scrolling down the list of subjects and click on the row to select the subject. When the subject is selected the subject name is highlighted.

Take a PWA measurement:

- While still in the "patient screen" open the study screen by clicking on "study" button. The screen will allow you enter the study details and to proceed to "Capture data"
- Click the "radial" check box
- Enter the diastolic and systolic blood pressure values that have been obtained from the cuff automated sphygmomanometer
- Click on the "Capture Data" button

- The tonometer should be placed perpendicular to the wrist and adjustments to the position should be made until a strong, accurate and reproducible waveform is displayed in the “Signal detail” window.
- When you are satisfied that you have a good reading, press the “Space Bar” on the key board.

You must have a minimum of 12 seconds of signal for the data to be captured. The last 2 seconds of waveforms will be deleted, allowing sufficient time to remove the tonometer for the wrist to activate the capture of data.

Examine the report for quality control:

After you have completed the data capture, the “report screen” will be automatically displayed. Two types of “Report Screen” are available, Clinical and detailed Screen, and each may be selected by clicking on the “clinical” or “detailed” tab. It is important to check the quality control to ensure that your measurement has been recorded with sufficient quality. The quality control indices are provided on the “detailed” report screen”. When the figures appear in green they are within the limits set using the configuration setting. When they appear in red they are outside theses limits. The measurement will be accepted if they are 2 red figures or less on the detailed screen.

For DVP, the indices recorded were heart rate (HR), reflective index (RI), stiffness index (SI) and peak-to-peak time (PPT). For PWA, the indices recorded were peripheral systolic (SBP), diastolic (DBP) and mean (MBP) blood pressure, heart rate, peripheral augmentation index (PAIx), central systolic (CSp) and diastolic blood pressure (CDp), central augmentation index (CAIx), Pressure at T1 (P1) and time to first peak (T1). The readings will be recorded on a form shown in and an average of the readings was used for analysis.

Appendix 7.14 Erythrocyte lipids

Erythrocyte lipids were measured in order to determine the omega-3 index as a sensitive marker of CV risk, as well as compliance, in addition to the capsules count. Blood was collected in 4.5 ml EDTA tubes and centrifuged at 1300 g, 4ºC for 15 min .The pellet (erythrocytes) was kept in the fridge 3 to 5 days before being treated according to the following protocol:.

Lipid extraction

Erythrocytes lipids: The buffy coat was removed, and the erythrocytes washed three times with 5 volumes of cold saline (0.89%) for 10 min at 900 g, 4ºC. 0.5 ml erythrocytes were pipetted into large 25 ml glass-stoppered centrifuge tubes and 0.5 ml distilled water added. The contents were vortex-mixed to haemolyse the erythrocytes. 5.5 ml chilled isopropanol were then added slowly, and the tube was vortexed thoroughly again. After 15 min storage at 4ºC, 3.5 ml chilled chloroform were added and the contents mixed thoroughly. At the end of another 30 min at 4ºC, the samples were centrifuged at 1500 g for 15 min at 4ºC. The supernatant was collected and stored in glass tubes at -40ºC until GC analysis.

Methyl ester preparation

Lipid extracts were treated in 3 batches; lipid extracts from the same subjects were treated at the same time.

Approximately 1 ml of each thawed lipid extract was transferred to a labelled GC vial and evaporated in an Eppendorff concentrator at 45 ºC for ~1 h. Each extract was then re-dissolved in 100 µl hexane, and 50 µl $NaOCH_3$ added to neutralise acidity. The content was thoroughly mixed and 1 ml hexane was added. $CaCl_2$ was then added in order to absorb any remaining water and the vials were covered and allowed to stand 1 h at RT. Samples were transferred to new GC vials, and evaporated for 20 min at RT. The final extracts were re-dissolved in 50 µl hexane and transferred to inserts in GC microvials for GC analysis.

GC conditions

Fatty acid methyl esters were separated on an Agilent 6890 Gas Chromatograph (Agilent Technologies) fitted with a flame ionization detector with a 25 m BP75 capillary column. The injection volume was 2 µL, the temperature was 160ºC for 4 min and then rose to 200ºC in 10 min (gradient of 12ºC/min).